

EXHIBIT A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Illana Gozes et al.

Application No.: 10/748,765

Filed: December 29, 2003

For: METHODS OF TREATING
AND/OR PREVENTING
AUTOIMMUNE DISEASES

Customer No.: 20350

Confirmation No. 8714

Examiner: C. M. Woodward

Technology Center/Art Unit: 1647

DECLARATION OF DR. ILLANA GOZES
UNDER 37 C.F.R. §1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Illana Gozes, Ph.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I am currently a Professor of clinical biochemistry at Tel Aviv University I am Director of the Adams Super Center for Brain Studies & Levie- Edersheim-Gitter Institute for Functional Brain Imaging, also at Tel Aviv University. I have been the incumbent of the Lily and Avraham Gildor Chair for the Investigation of Growth Factors at Tel Aviv University, since 1997. I am also the Chief Scientific Office and Director at Allon Therapeutics, Inc. in Vancouver, Canada.

I received a Ph.D. from The Weizmann Institute of Science in 1979, and was a Haim Weizmann Postdoctoral Fellow at the Massachusetts Institute of Technology from 1979-

1980. I was a Research Associate and Visiting Scientist at the Salk Institute and the Scripps Clinic and Research Foundation from 1981-1982. I was a Senior Scientist/Associate Professor at The Weizmann Institute of Science from 1982-1989. I was a visiting scientist in developmental neurobiology at NICHD, NIH from 1989-1990. My affiliation with Tel Aviv University began in 1990. I was a Fogarty-Scholar-in-Residence at NIH from 1995-1996 and an adjunct scientist in developmental neurobiology at NIH from 2003-2004.

3. I have received a number of scientific awards and prizes, including the Juludan Prize and the Teva Founders Prize for exceptional scientific studies and the Bergmann Prize and the Neufeld award for outstanding/leading US-Israel BSF grant proposals. I am currently Editor-in-Chief of The Journal of Molecular Neuroscience and I currently sit on the editorial boards of the American Journal of Alzheimer's Disease, the International Journal of Peptide Research & Therapy and the journal Peptides. I am an author on more than 205 research papers and am an author or co-author of numerous reviews and book chapters. A copy of my curriculum vitae is attached hereto as Exhibit B and includes a list of selected publications.

4. The present invention is a method of treating multiple sclerosis (MS) by administering a therapeutically effective amount of an ADNF III peptide to a subject in need of such treatment. The treatment includes administration of a peptide that comprises the core active site sequence of ADNF III, *i.e.*, the amino acid sequence NAPVSIPQ known as "NAP." NAP is the smallest peptide that exhibits the same activity as full-length ADNF III. Use of all D-amino acid NAP peptides are also claimed.

5. I have read and am familiar with the contents of this patent application. In addition, I have read an Office Action, dated March 6, 2007, received in the present case. It is my understanding that the Examiner alleges that the full scope of the claimed invention is not enabled by the specification. Specifically, the Office Action alleges that the disclosed genus of ADNF III peptides with core active site sequence of SEQ ID NO:2 is unlikely to have the disclosed biological activity (Office Action at page 5); that all D-amino acid ADNF III peptides are unlikely to have biological function (Office Action at page 6); and that undue experimentation is required to practice the invention using the claimed genus of ADNF III

peptides because the specification lacks guidance/direction to use the claimed methods and sufficient working examples for treatment of MS (Office Action at page 8).

6. This declaration is provided to demonstrate that the specification teaches full use of the claimed methods. The specification, in combination with the knowledge in the art at the time of filing, provides sufficient guidance to allow one of skill to use the claimed methods to treat MS in a subject, with only routine experimentation, using an ADNF III peptide that comprises the core active site of ADNF III, *i.e.*, NAP. In particular, this declaration provides evidence that an assay system for measurement of the treatment activity of ADNF peptides against MS, the myelin-oligodendrocyte glycoprotein (MOG)-induced chronic experimental autoimmune encephalomyelitis (EAE) model, is an art accepted model of multiple sclerosis. This declaration is provided to demonstrate that the genus of proteins that comprise the ADNF III core sequence can be used in the claimed methods and that those of skill can identify active members of the genus of ADNF III proteins and distinguish inactive ADNF III proteins from active proteins using the EAE model in the specification. This declaration also provides evidence that all-D amino acid ADNF III core peptides will likely have activity in the claimed methods.

7. At the time of filing the core sequence of the ADNF III peptide, Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2), had been identified as the smallest active sequence of the larger full-length ADNF III peptide. The ADNF III core sequence was sufficient for biological activity as measured in, *e.g.*, neuroprotection assays. While the amino acids surrounding the ADNF III core sequence can vary, the eight amino acids of the ADNF III core sequence are invariant. This application discloses that MS can be treated by ADNF peptides comprising the ADNF III core sequence. As with neuroprotective activity, the ADNF III core sequence is sufficient for activity against MS and that activity can be maintained with additional amino acids can be added to the invariant ADNF III core sequence. Finally, the specification provides an assay system for measurement of the activity of ADNF peptides against MS: the MOG-induced chronic EAE model. (Specification at pages 30-31.)

8. I have read the references cited by the Office Action as supporting the rejection for alleged lack of enablement of the recited genus of ADNF III peptides, *e.g.*, Tischler *et al.* (US Patent 5,194,596); Kopchick (US Patent 5,350,836); Skolnick *et al.* TIBS 18:34-39 (2000) and Smith *et al.*, (Nature Biotechnology 15:1222-1223 (1997)). I do not agree with the analysis of the Office Action. Tischler *et al.* and Kopchick support the usefulness of sequence alignments for identifying amino acid residues that, if mutated, are most likely to affect protein activity. Kopchick also demonstrate that results of amino acid substitutions can be predicted by those of skill. Skolnick and Smith describe problems of predictive biology that do not apply to the claimed invention.

9. Tischler *et al.* at column 2, line 46, disclose homology between a vascular endothelial growth factor (VEGF) and a platelet derived growth factor (PDGF). Tischler *et al.* disclose conserved cysteine residues on the two growth factor proteins and swapped homologous domains. Tischler *et al.* showed that certain conserved regions of the growth factor proteins were responsible for mitogenic activity toward vascular smooth muscle cells or vascular endothelial cells. Tischler *et al.* then showed a hybrid molecule that included the portions of both VEGF and PDGF molecules would have mitogenic activity that correlated with the originating molecule of the conserved region. That is, a hybrid molecule with a conserved region from the VEGF protein would be mitogenic toward vascular endothelial cells. Thus, Tischler *et al.* demonstrate that those of skill can identify important functional regions of a protein and manipulate recombinant proteins to have a desired function. In my opinion, Tischler *et al.* demonstrates that modification of proteins, including the ADNF III protein, can be done by those of skill with only routine experimentation.

10. Kopchick *et al.* disclose a proposed structure for a conserved region of amino acids in a growth hormone protein. (Kopchick *et al.* at column 9.) Kopchick *et al.* identified amino acid 119, a glycine residue, as a target for mutagenesis that would inactivate the growth factor. Based on the known characteristics of amino acids Kopchick *et al.* were able to predict which amino acids could be substituted for the glycine at amino acid 119 and provide a desired growth inhibitory phenotype. Thus, Kopchick *et al.* demonstrate that generation of nonfunctional proteins by mutagenesis can be anticipated by those of skill, particularly when

functional, structural and sequence information is known. In my opinion, Kopchick *et al.* demonstrates that modification of proteins, including the ADNF III protein, can be done by those of skill with only routine experimentation.

11. Both Skolnick and Smith attempt to demonstrate that methods to identify a protein function based solely on comparison of an unknown amino acid sequence to known amino acid sequences with known functions are "inconsistent" (Smith) or "inadequate" (Skolnick). The Smith and Skolnick references discuss only the problems of assigning function to a previously unknown protein based solely on sequence comparisons and lacking experimental evidence of function. This type of analysis is not relevant to the claimed invention which recites use of polypeptides that comprise the invariant ANDF III core sequence. Example 1 of the specification provides experimental evidence of the anti-MS activity if the ADNF III peptide. As the function of the ADNF III peptide has been experimentally determined, and assays to measure those function are known and routine, those of skill are able to identify functional variants of the ADNF III peptide of SEQ ID NO:2. Thus, the concerns raised by Smith and Skolnick do not apply to the claimed invention.

12. MOG-induced chronic EAE model is an art-accepted model for MS. To induce MS-like symptoms of axonal damage and demyelination, EAE was induced by immunization with the peptide encompassing amino acids 35-55 of rat MOG. A previous study by Offen *et al.* investigated the possible role of axonal susceptibility and resistance to reactive oxygen species (ROS) in the pathogenesis of EAE. (Offen *et al. J Mol Neurosci.* 15(3):167-76 (2000), submitted as Exhibit C). Offen *et al.* demonstrated that clinical manifestations of MS were apparent in the MOG-induced chronic EAE model. Clinical manifestations included loss of tail tonicity, partial hind-limb paralysis, and complete hind-limb paralysis. *See, e.g., Offen et al., Figure 1, page 170.* Offen *et al.* also demonstrated that after immunization with MOG, the immune response to the protein included T cell proliferation. *See, e.g., Offen et al., Figure 3, page 172.*

13. The specification demonstrates the effect of ADNF III peptides on the MOG-induced chronic EAE model at paragraphs 103-106. EAE was induced by immunization

with the peptide encompassing amino acids 35-55 of rat MOG. Synthesis was carried out by the Weizmann Institute Synthesis Unit using a solid-phase technique, on a peptide synthesizer (Applied Biosystems Inc., Foster City, CA City). Six weeks old C57/b mice (Tel-Aviv University) were injected (subcutaneous) in the flank with a 200µl emulsion containing 300µg MOG peptide in complete Freund adjuvant (CFA) and 500µg Mycobacterium tuberculosis (Sigma Israel). An identical booster immunization was given on the other flank one week later. Ten days following the encephalitogenic challenge, the MOG-treated mice were observed daily and the clinical manifestations of EAE were measured by the following score: 0 = no clinical symptoms; 1 = loss of tail tonicity; 2 = partial hind limb paralysis; 3 = complete hind limb paralysis; 4 = partial frontal limb paralysis; 5 = complete frontal limb paralysis; 6 = death.

For treatment, NAP was administered (intranasal) 0.1 microgram/mouse in a mixture containing 7.5 mg/ml sodium chloride, 1.7 mg/ml citric acid monohydrate, 3.0 mg/ml disodium phosphate dehydrate and 0.2 mg/ml of a 50% benzalkonium chloride solution. The nasal administration was given daily, 1 hour after MOG injection and was continued and given once a day, 1 hour prior to testing. Control animals received the above mixture without NAP. In the example here, NAP's daily treatment began 10-14 days prior to the MOG injection.

Results showed that NAP significantly improved the clinical outcome of the animals, day 11 on, $P < 0.01$, t-test (Figure 1 of the specification).

14. An additional experiment included proliferative T-cell response performed as described by Offen *et al.*, *supra*. Results indicated that NAP inhibited the immune response (cell proliferation, Fig. 2 of the specification) *in vivo* as the proliferative response of splenocytes was much reduced ($P < 0.01$) in the mice treated with NAP as compared to untreated mice. Furthermore, addition of MOG resulted in increased proliferation in the splenocytes of untreated animals, even at 2 micrograms/well of MOG, $P < 0.05$). In contrast, even at 25 micrograms MOG, the proliferative response NAP treated animals did not increase.

15. Using the art-accepted model of MOG-induced chronic EAE, ADNF III polypeptides have been shown to reduce conditions associated with MS. Specifically, treatment with NAP resulted in improved clinical outcome (e.g., lessened limb paralysis), when compared to control mice that did not receive NAP. Treatment with NAP also reduced the immune

response to the administered MOG, as measured by T-cell proliferation. The MOG-induced chronic EAE model system can be used to test the function of ADNF III peptides in treatment of MS.


16. The Office Action appears to allege that identification of functional ADNF III polypeptides is unpredictable because of use of a peptide comprising the ADNF III core sequence as an inactive control in the PCT publication WO/20002785. I supervised the experiments disclosed in the PCT publication. We first cloned a large fragment of a nucleic acid that encodes the ADNF III polypeptide. After deciding to identify the smallest portion of the ADNF III protein necessary for activity, we made deletions in the coding sequence and made progressively smaller ADNF III peptides for testing. We used routine molecular biology techniques or peptide synthesis techniques to make the series of ADNF III polypeptides for testing. We used the neuroprotection assays to determine the activity of each ADNF III peptide. Not every ADNF III peptide tested had activity, but using the combination of routine protein synthesis techniques and neuroprotection assays, we were able to 1) identify the smallest ADNF III peptide required for neuroprotective activity and 2) efficiently distinguish between active and inactive ADNF III peptides. Similar experiments can be carried out by those of skill to determine the activity of ADNF III peptides using the art-accepted model of MOG-induced chronic EAE described above. Thus, based on the teaching of the specification, those of skill will be able to efficiently distinguish between MS-inhibitory and non-inhibitory ADNF III peptides.

17. The Office Action alleges that there is no teaching in the specification that would support any biological function for a protein comprising an all D-amino acid ADNF III core sequence (SEQ ID NO:2). Office Action at page 6. At the time of filing, it was known that all D-amino acid versions of ADNF peptides, including NAP and the related protein SAL (the active core sequence of the ADNF I protein), were active in neuroprotection assays. *See, e.g.*, WO 01/12654, included as Exhibit D, at page 46, line 23 through page 47, line 21 and Figures 1 and 2. Exhibit E provides experimental evidence that all D-amino acid SAL provided a treatment benefit when administered to mice with MOG-induced chronic EAE. As indicated above, MOG-induced chronic EAE is art-accepted model of MS. The mice that did not receive

D-SAL had progressive clinical impairment, as expected. See, Figure in Exhibit E. In contrast, in mice that received D-SAL, clinical impairment progressed more slowly. The effect is especially apparent in MOG-immunized mice treated with 2 micrograms of D-SAL. Thus, as assessed by the art accepted model, D-SAL administration provided clinical benefit to the MOG-immunized mice. D-NAP and D-SAL have similar effects when assessed in a number of assays, including neuroprotection assays. See, *e.g.*, PCT publication WO/20002785. Therefore, based on the above-described data, it is my scientific opinion that all D-amino acid NAP will have an effect similar to all D-SAL when administered to MOG-immunized mice in the EAE art-accepted model for MS.

18. In view of the foregoing, it is my scientific opinion that one of skill in the art would be able to practice the claimed invention with, at most, routine experimentation. The specification, therefore, fully enables the methods of the invention.

Date: September 3, 2007

By:  _____

Illana Gozes, Ph.D.

EXHIBIT B

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Ilana Gozes		POSITION TITLE Professor of Clinical Biochemistry, The Lily and Avraham Gildor Chair for the Investigation of Growth Factors, Director the Adams Super Center for Brain Studies, Tel Aviv University; Chief, Scientific Officer, Allon Therapeutics Inc.		
eRA COMMONS USER NAME IGOZES@POST.TAU.AC.I				
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)				
INSTITUTION AND LOCATION		DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Tel Aviv University (TAU)		B.Sc.	1973	Biology, Life Sciences
Feinberg Graduate School, Weizmann Institute		Ph.D.	1979	Neurobiology
Massachusetts Institute of Technology (MIT)			1979-1980	Neuroendocrinology
The Salk Institute & Scripps Clinic			1981-1982	Molecular Neurobiology

A. Positions and Honors.**Positions and Employment**

- 1982-1987 Senior Scientist of Hormone Research, Weizmann Institute of Science
1987-1989 Associate Professor of Hormone Research, Weizmann Institute of Science
1989-1990 Visiting Scientist in Developmental Neurobiology, NICHD, NIH.
1990-1993 Associate Professor of Chemical Pathology, Tel Aviv University (TAU)
1993- Professor of Clinical Biochemistry, Tel Aviv University (TAU)
2001-2003 Chief Executive Scientist, Chair of the Board; Allon Therapeutics, Inc. La Jolla, CA, USA
2003-2004 Chief Scientific Officer, Vice Chair of the Board, Allon Therapeutics, interim CEO (summer 2004)
2004- Chief Scientific Officer and Director; Allon Therapeutics, Inc. Vancouver, BC, Canada
2006- Director, Adams Super Center for Brain Studies & Edersheim Levi-Gitter fMRI Inst. At TAU

Other Experience and Professional Memberships

- 1988-1989 Visiting Scientist in Molecular Genetics, NICHD, NIH
1989-2003 Invited Guest Scientist in Developmental Neurobiology (Summers) NICHD, NIH
1993-1994 Chair, Department of Chemical Pathology, Tel Aviv University (TAU)
Chair, Forum of Preclinical Department Heads, Sackler School of Medicine, TAU
1994-1995 Chair, Department of Clinical Biochemistry, Tel Aviv University
1995-1996 Fogarty Scholar-in-Residence, NIH
1997- The Lily and Avraham Gildor Chair for the Investigation of Growth Factors, Tel Aviv University
1997- Senate committee for TAU Masters Degrees (to 2001), The Israeli Neuroscience Society (to 1999).
The Sackler Faculty of Medicine: R&D Committee. Graduate School Committee (-2001)
1998- The Sackler Faculty of Medicine: Head, International Advisory Committee (-2001)
Member, Committee of School Heads (-2000)
1998- Co-Editor-in-Chief, 2000- Editor-in-Chief, The Journal of Molecular Neuroscience, Humana Press
Member: the scientific advisory board: Institute for the study of aging (ISOA), NY, USA
1999-2003 Member of the Sackler Faculty Pre-Clinical Appointment and Promotion Committee
2000- Executive guest editor: Current pharmaceutical Design, Clore Scholars Board Member (-2003)
2001- Co-director, the TAU-Sackler Med. School-NIH student program in Women Health
Chair of the organizing committee Neuropeptides 2001 Meeting (Israel), Co-Chair, American Meeting
2003-2004 Adjunct Scientist in Developmental Neurobiology, NICHD, NIH
2002- TAU Senate Committee for postdoctoral fellows (-2003), Sackler Faculty of Medicine, Grants Committee (-2006); Co-chair: the American Neuropeptides Summer Meeting.
2003 Selection committee for Gottlieb Award (ISOA, NY, USA), VIP and Related Peptide, Scientific Advisory Committee; Secretary General the European Neuropeptide Club
2005- Editorial Board, American Journal of Alzheimer's Disease; International J of Peptide Res. & Therap.
International Advisory Committee, the VIP PACAP and Related Peptides Meeting
Member, Scientific Advisory Committee: The United State Israel Binational Science Foundation (-200
2006- Editorial Board, Peptides, member Professorial Chairs Committee, Sackler Faculty of Medicine, Member, TAU Committee on Faculty Equal Rights, TAU Board of Governors

Honors (selected)

- 1977 Landau Prize (Miphal Ha-pals) Excellent Ph.D., Katzir Fellowship
1978-1980 Chaim Weizmann Post-Doctoral Fellowship, EMBO short-term fellowship (1978)
1982-1985 The Bergmann Memorial Fund Research Prize, for excellent BSF grant (nationwide)
1983-1989 The first Incumbent of Samuel O. Freedman Career Development Chair
1991 The Judan Prize for outstanding research achievements
1993 Teva Founders Prize for opening new horizons in medical research in Israel
1994 Fogarty International Scholar
1997- The Lily and Avraham Gildor Chair for the Investigation of Growth Factors
2000-2003 The Neufeld Grant Award for excellent BSF grant application in health sciences, nation-wide
2000 Best scientific work award: The Israeli Society for Laboratory Sciences
2003-2004 The Mariana and George Sayer grant, HIV and Parkinson (Tel Aviv Univ.)
2003/4 Best student best paper, Brain Research (Dr. Albert Pinhasov), Poster award – VIP-PACAP meeting (student, Inna Divinski); Dan David Scholarship Student (Shmuel Mandel, student)
2006 Boaz Moav Prize for Develop. Biology (Switzerland Inst at Tel Aviv Univ (S. Mandel, student))

B. Selected peer-reviewed publications (In chronological order), (from >197 reviewed publications)

1. I Gozes, H Schmitt, UZ Littauer Translation in vitro of rat brain messenger RNA coding for tubulin and actin. *Proc Natl Acad Sci USA* 1975; 72:701-705.
2. I Gozes, MD Walker, AM Kaye, UZ Littauer Synthesis of tubulin and actin by neuronal and glial nuclear preparations from developing rat brain. *J Biol Chem* 1977;252:1819-1825.
3. I Gozes, UZ Littauer Tubulin microheterogeneity increases with rat brain maturation. *Nature* 1978;276: 411-4
4. I Gozes, KJ Sweadner Multiple forms of tubulin are expressed by a single neuron. *Nature* 1981;294:477-480
5. I Gozes, CJ Barnstable Monoclonal antibodies that recognize discrete forms of tubulin. *Proc Natl Acad Sci USA* 1982; 79:2579-2583.
6. Y Gozes, MA Moskowitz, TB Strom, I Gozes Conditioned media from activated lymphocytes maintain sympathetic neurons in culture. *Dev Brain Res* 1983;6: 93-97.
7. M Bodner, M Fridkin, I Gozes VIP and PHM-27 sequences are located on two adjacent exons in the human genome. *Proc Natl Acad Sci USA* 1985;82:3548-3551.
8. F Baldino, S Fitzpatrick-McElligott, I Gozes, JP Card Localization of VIP and PHI-27 messenger RNA in rat thalamic and cortical neurons. *J Mol Neurosci* 1989;1:199-207.
9. E Giladi, Y Shani, I Gozes The complete structure of the rat VIP-gene. *Mol Brain Res* 1990;7: 261-267.
10. I Gozes, SK McCune, J Jacobson, D Warren, TW Moody, M Fridkin, DE Brenneman An antagonist to vasoactive intestinal peptide: effects on cellular functions in the central nervous system. *J Pharmacol Exp Ther* 1991;257:959-966.
11. P Gressens, JM Hill, I Gozes, M Fridkin, DE Brenneman Growth factor function of vasoactive intestinal peptide in whole cultured mouse embryos. *Nature* 1993; 362:155-158.
12. I Gozes, J Glowa, DE Brenneman, SK McCune, E Lee, H Westphal Learning and sexual deficiencies in transgenic mice carrying a chimeric vasoactive intestinal peptide gene. *J Mol Neurosci* 1993; 4:185-193.
13. I Gozes, A Bardea, A Reshef, R Zamostiano, S Zhukovsky, S Rubinstein, M Fridkin, DE Brenneman Novel Neuroprotective strategy for Alzheimer's disease: inhalation of a fatty neuropeptide. *Proc Natl Acad Sci USA* 1996;93:427-32.
14. DE Brenneman, I Gozes A femtomolar-acting neuroprotective peptide. *J Clin Invest* 1996;97: 2299-307.
15. M Bassan, R Zamostiano, A Davidson, A Pinhasov E Giladi, O Perl, H Bassan, C Blatt, G Gibney, G Glazner, DE Brenneman, I Gozes Complete cDNA sequence of a novel protein containing a femtomolar-activity-dependent neuroprotective peptide. *J Neurochem* 1999;72:1283-93.
16. I Gozes, O Perl, E Giladi, A Davidson, O Ashur-Fabian, S Rubinstein, M Fridkin Mapping the active site in vasoactive intestinal peptide to a core of four amino acids: neuroprotective drug design. *Proc Natl Acad Sci USA* 1999;96: 4143-48.
17. P Gressens, L Besse, P Robberecht, I Gozes, M Fridkin, P Evrard Neuroprotection of the developing brain by systemic administration of vasoactive intestinal peptide derivatives. *J Pharmacol Exp Ther* 1999;288:1207-13
18. D Offen, YG Sherki, E Melamed, M Fridkin, DE Brenneman, I Gozes Vasoactive intestinal peptide (VIP) protects from dopamine toxicity: relevance to Parkinson's disease. *Brain Res.* 2000; 854:257-62.
19. I Zemlyak, S Furman, DE Brenneman I Gozes A novel peptide NAP prevents death in enriched neuronal cultures. *Regulatory Peptides* 2000;96:39-43.

20. I Gozes, E Giladi, A Pinhasov, A Bardea, DE Brenneman Activity-dependent neurotrophic factor: intranasal administration of femtomolar-acting peptides improve performance in a water maze. *J Pharmacol Exp Ther* 2000;293:1091-98.
21. O Blondel, C Collin, B McCarran, S Zhu, R Zamostiano, I Gozes, DE Brenneman, RDG McKay A GliA-derived signal regulating neuronal differentiation. *J Neurosci* 2000; 20:8012-20.
22. R A Steingart, B Solomon, D E Brenneman, M Fridkin, I Gozes VIP and peptides related to activity-dependent neurotrophic factor protect pc12 cells against oxidative stress. *J Mol Neurosci.* 2000;15:137-45
23. L Beni-Adani, I Gozes, Y Cohen, Y Assaf, RA Steingart, DE Brenneman, O Elzenberg, V Trembovler, E Shohami A peptide derived from activity-dependent neuroprotective protein (ADNP) ameliorates injury response in closed head injury mice. *J Pharmacol Exp Ther* 2001; 296:57-63.
24. R Zamostiano, A Pinhasov, E Gelber, R A Steingart, E Seroussi, E Giladi, M Bassan, Y Wollman, H J Eyre, JC Mulley, D E Brenneman and I Gozes Cloning and Characterization of the Human Activity-Dependent Neuroprotective Protein (ADNP). *J Biol Chem* 2001;276:708-14.
25. CY Spong, DT Abebe, I Gozes, DE Brenneman and JM Hill Prevention of fetal demise and growth restriction in a mouse model of fetal alcohol syndrome. *J Pharmacol Exp Ther* 2001; 297, 774-9.
26. O Ashur-Fabian, E Giladi, S Furman, RA Steingart, Y Wollman, M Fridkin, DE Brenneman, I Gozes Vasoactive intestinal peptide and related molecules induce nitrite accumulation in the extracellular milieu of rat cerebral cortical cultures. *Neurosci Lett* 2001; 307:167-70.
27. I Gozes Neuroprotective peptide drug delivery and development: potential new therapeutics. *Trends in Neurosci.* 2001;24:700-5.
28. J Romano, L Beni-Adani, OL Nissenbaum, DE Brenneman, E Shohami, I Gozes A single administration of the peptide NAP induces long-term protective changes against the consequences of head injury: gene Atlas array analysis. *J Mol Neurosci* 2002;18: 37-45.
29. RR Leker, A Teichner, R Nussen, N Grigoriadis, Y Cohen, H Ovadia, DE Brenemann, M Fridkin, E Giladi, J Romano, I Gozes NAP, a femtomolar-acting peptide, protects the brain against ischemic injury by reducing apoptotic death. *Stroke* 2002;33:1085-92.
30. R Zaltzman, SM Beni, E Giladi, A Pinhasov, RA Steingart, J Romano, E Shohami, I Gozes Injections of the neuroprotective peptide NAP to newborn mice attenuate head-injury related dysfunction in adults. *Neuroreport* 2003;14:481-4.
31. A Pinhasov, A.M Mandel, Torchinsky, E Giladi, Z Pittel, AM Goldswelg, SJ Servoss, DE Brenneman, I Gozes Activity-Dependent Neuroprotective Protein: a novel gene essential for brain formation. *Brain Res Dev Brain Res* 2003;144:83-90
32. O Ashur-Fabian, Y Segal-Ruder, E Skutelsky, RA Steingart, E Giladi, D E Brenneman, I Gozes The neuroprotective peptide NAP inhibits the aggregation of the beta-amyloid peptide. *Peptides* 2003;24: 1413-23
33. DE Brenneman, CY Spong, HM Hauser, D Abebe, A Pinhasov, T Gollan, I Gozes Protective peptides that are orally active and mechanistically non-chiral. *J Pharmacol Exp Ther* 2004;309:1190-7.
34. RN Alcalay, E Giladi, CG Pick, I Gozes Intranasal administration of NAP, a neuroprotective peptide, decreases anxiety-like behavior in aging mice in the elevated plus maze. *Neurosci Lett* 2004;361:128-31.
35. R Zaltzman A Alexandrovich, SM Beni, V Trembovler, E Shohami, I Gozes Brain injury-dependent expression of activity-dependent neuroprotective protein. *J Mol Neurosci* 2004; 24:181-7.
36. I Divinski, L Mittelman, I Gozes A femtomolar-acting octapeptide interacts with tubulin and protects astrocytes against zinc intoxication *J Biol Chem* 2004;279:28531-8.
37. M Zusev, I Gozes Differential regulation of activity-dependent neuroprotective protein in rat astrocytes by VIP and PACAP. *Regul Pept* 2004;123:33-41.
38. S Furman, RA Steingart, S Mandel, JM Hauser, DE Brenneman, I Gozes. Subcellular localization and secretion of activity-dependent neuroprotective protein in astrocytes. *Neuron Glia Biology* 2004;1:193-9.
39. S Furman, JM Hill, I Vulić, R Zaltzman, JM Hauser, DE Brenneman, I Gozes Sexual dimorphism of activity-dependent neuroprotective protein in the mouse arcuate nucleus. *Neurosci Lett* 2005;373:73-8.
40. VL Smith-Swintosky, I Gozes, DE Brenneman, MR D'Andrea, CR Plata-Salamán. Activity-dependent neurotrophic factor-9 and NAP promote neurite outgrowth in rat hippocampal and cortical cultures. *J Mol Neurosci.* 2005;25:225-38.
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42. R Zaltzman, A Alexandrovich, V Trembovier, E Shohami, I Gozes. The Influence of the peptide NAP on Mac-1-deficient mice following closed head injury. *Peptides*. 2005; 26:1520-7.
 43. L Vlisochek, RA Steingart, I Vulih-Shultzman, R Klein, E Priel, I Gozes, M Cohen-Armon. PolyADP-ribosylation is involved in neurotrophic activity. *J Neurosci*. 2005;25:7420-8.
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C. Research Support: Ongoing Research Support

- October 2003-September 2007 Allon Therapeutics through Ramot (PI). Goals: Understanding NAP and pipeline peptide products toward neuroprotective drug development
- October 2004- September 2008- The US-Israel Binational Science Foundation (PI), together with Drs. Peng Y. Loh and Dr. Joanna Hill. Understanding ADNP processing into smaller molecules.
- October 2003-September 2007- The Israel Science Foundation (PI). ADNP in CNS development
- October 2004- NIA contract. Ongoing contract for NAP toxicology (consultant)
- March 2006 – Nofar – Governmental-Industrial Support (Cancer Research)
- Completed Research Support (selected)**
- October 1999- September 2004 NIA contract N01-AG-9-2117 NAP toxicology (consultant)
- October 1999-September 2003 The Israel Science Foundation (PI). NAP related peptides
- 1999 – 2002, The Institute for the Study of Aging (ISOA) (PI) Femtomolar-Acting Neuroprotective Peptides: Application for Inhibition of Neurodegenerative Disease; 2003 – 2005 (PI) NAP toward phase I clinical trials.
- October 2000-September 2003 BSF (PI), together with Dr. DE Brenneman (NIH). Understanding ADNP

EXHIBIT C

Mice Overexpressing Bcl-2 in Their Neurons Are Resistant to Myelin Oligodendrocyte Glycoprotein (MOG)-Induced Experimental Autoimmune Encephalomyelitis (EAE)

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Abstract

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) characterized by destruction of myelin. Recent studies have indicated that axonal damage is involved in the pathogenesis of the progressive disability of this disease. To study the role of axonal damage in the pathogenesis of MS-like disease induced by myelin oligodendrocyte glycoprotein (MOG), we compared experimental autoimmune encephalomyelitis (EAE) in wild-type (WT) and transgenic mice expressing the human bcl-2 gene exclusively in neurons under the control of the neuron-specific enolase (NSE) promoter. Our study shows that, following EAE induction with pMOG 35–55, the WT mice developed significant clinical manifestations with complete hind-limb paralysis. In contrast, most of the NSE-bcl-2 mice (16/27) were completely resistant, whereas the others showed only mild clinical signs. Histological examination of CNS tissue sections showed multifocal areas of perivascular lymphohistiocytic inflammation with loss of myelin and axons in the WT mice, whereas only focal inflammation and minimal axonal damage were demonstrated in NSE-bcl-2 mice. No difference could be detected in the immune potency as indicated by delayed-type hypersensitivity (DTH) and T-cell proliferative responses to MOG. We also demonstrated that purified synaptosomes from the NSE-bcl-2 mice produce significantly lower level of reactive oxygen species (ROS) following exposure to H₂O₂ and nitric oxide (NO) than WT mice. In conclusion, we demonstrated that the expression of the anti-apoptotic gene, bcl-2, reduces axonal damage and attenuates the severity of MOG-induced EAE. Our results emphasize the importance of developing neuroprotective therapies, in addition to immune-specific approaches, for treatment of MS.

Index Entries: Myelin oligodendrocyte glycoprotein (MOG); experimental autoimmune encephalomyelitis (EAE); multiple sclerosis (MS), bcl-2.

^{*}D.O. and J.F.K. contributed equally.

Introduction

Multiple sclerosis (MS) is a chronic disabling disease of the central nervous system (CNS), with relapsing-remitting or a chronic-progressive clinical manifestations. The etiology of MS has not yet been fully elucidated, but it is believed that immunological mechanisms are involved in disease initiation and progression (Stinissen et al., 1997). Although the major histological hallmark of MS lesions in the CNS is demyelination with destruction of the myelin sheath and death of oligodendrocytes, it has been proposed that mild to moderate axonal damage and loss occur in the late chronic progressive stage of the disease (Kornek and Lassmann, 1999; De Stefano et al., 1999; Trapp et al., 1999; Silber and Sharief, 1999). It was also found that antibodies against amyloid precursor protein (APP), known to be a sensitive marker of axonal damage, are present within acute MS lesions and in the active borders of less-acute plaques (Ferguson et al., 1997). Furthermore, magnetic resonance spectroscopy measurements of N-acetyl-aspartate (NAA), a compound found exclusively in neurons and their projections in the adult brain, indicate the presence of axonal damage within the lesions and the surrounding white matter (Davie et al., 1995). Pathological studies in MS patients have demonstrated a high frequency of terminal axonal ovoids, indicating axonal transection that correlated with irreversible neurological impairment (Fu et al., 1998; Trapp et al., 1998). Recent studies have proposed that oxidative stress, triggered by cytokines and inflammatory mediators, may also contribute to the axonal damage, neuronal loss, and brain atrophy following demyelination (Trapp et al., 1998; van Walderveen et al., 1999; Zhu et al., 1999).

In this study, we investigated the possible role of axonal susceptibility and resistance to reactive oxygen species (ROS) in the pathogenesis of MS, using experimental autoimmune encephalomyelitis (EAE), an accepted animal model of MS. To evaluate the extent of this resistance to ROS, we used NSE-bcl-2 transgenic mice that overexpress the human bcl-2 gene exclusively in their neurons, under the control of neuron-specific enolase promoter (Farlie et al., 1995). Previous studies have demonstrated that these mice contain increased number of neurons owing to increased resistance to neuronal death during development (Farlie et al., 1995; Bernard et

al., 1997; Coleman et al., 1999). Increased resistance to cell death was also observed during induced ischemia (Martinou et al., 1994). In addition, several studies have shown that these NSE-bcl-2 mice are also resistant to experimentally induced neurodegenerative diseases such as Parkinson's disease (Offen et al., 1998; Yang et al., 1998), Alzheimer's disease (Saille et al., 1999), and amyotrophic lateral sclerosis (ALS; Kostic et al., 1997). We now demonstrate that the clinical features, as well as inflammation and axonal damage, in MOG-induced EAE are significantly attenuated in NSE-bcl-2 mice.

Materials and Methods

Mice

Male and female wild-type (WT) C57BL/6 (H-2^b) and NSE-Bcl-2/B6 transgenic mice were established and characterized in the laboratory of Dr. Ora Bernard (WEHI, Melbourne, Australia; Farlie et al., 1995). All mice used in these experiments were 2–3 mo old.

EAE Induction

EAE was induced by immunization with the peptide encompassing amino acids 35–55 of rat myelin oligodendrocyte glycoprotein (MOG). Synthesis was carried out by the Weizmann Institute Synthesis Unit, using a solid-phase technique on a peptide synthesizer (Applied Biosystems Inc., Foster City, CA). Mice were injected subcutaneously at one site in the flank with a 200 μ L emulsion containing 300 μ g MOG peptide in complete Freund adjuvant (CFA) and 500 μ g *Mycobacterium tuberculosis* (Sigma, Israel). An identical booster immunization was given at one site on the other flank 1 wk later. The mice were also injected with 300 ng pertussis toxin (PT, Sigma, Israel) in 500 μ L phosphate-buffered saline (PBS) in the tail vein immediately and again at 48 h after the first immunization. Following the encephalitogenic challenge, mice were observed daily and clinical manifestations of EAE were scored as previously described (Mendel et al., 1996).

Histopathology

Brains and spinal cords from WT and NSE-bcl-2 mice were dissected out 25 d after immu-

nization with pMOG 35-55, fixed in 10% buffered formalin, and embedded in paraffin. Five micron-thick sections were stained with Hematoxylin and Eosin (H&E). The bielschowsky's method was used for evaluation of the axonal population (Luna, 1968a). The Luxol Fast Blue (LFB) stain combined with an H&E stain was used for the assessment of myelin loss (Luna, 1968b).

Delayed-Type Hypersensitivity (DTH) Assay

Mice were injected with 100 μ L of an emulsion containing 150 μ g MOG peptide (pMOG 35-55) in CFA and 250 μ g *M. tuberculosis* in the foot pads. Nine days later, 20 μ L of PBS was injected into the left ear (LHS) and 20 μ L of 1.0 mg/mL pMOG 35-55 (20 μ g) or Purified Protein Derivative (PPD) of *M. tuberculosis* (Statens Serum Institute, Denmark) was injected to the right ear. The thickness of the ears was determined before and 24 h after injection into the ear using a micrometer (Mitutoyo, Japan).

T-Cell Proliferation Assay

Popliteal lymph-node cells (5×10^6) isolated from mice injected subcutaneously in the foot pads with 100 μ L of emulsion containing 150 μ g pMOG 35-55 in CFA with 250 mg of *M. tuberculosis*, were cultured in microtiter wells as previously described (Ben-Nun et al., 1981). The relevant peptide was added (0.5, 1.0, and 2 μ g/well) in triplicate cultures. The cultures were incubated for 48 h at 37°C in humidified air containing 7.5% CO₂. [³H]-thymidine (1 μ Ci/well) was added for the last 16 h of the incubation and the cultures were then harvested and counted using a Matrix 96 Direct beta counter (Packard Instr., Meriden, CT). The proliferative response is measured as the [³H]-thymidine incorporation expressed as mean counts per minute (CPM) of triplicate cultures.

Isolation of Synaptosome

Synaptosomal fractions were isolated from whole brains as previously described by Lebel et al. (1992). Briefly, brains were homogenized in 10 vol (w/v) of 0.32 M sucrose (ice-cold) utilizing a teflon/glass homogenizer. The nuclear fraction was removed by centrifugation at 1800g at 4°C for 15 min. The pellet was resuspended in HEPES buffer (120 mM NaCl, 2.5 mM KCl, 1.2 NaH₂PO₄,

0.1 mM MgCl₂, 5 mM NaHCO₃, 6 mM glucose, 1 mM CaCl₂, 10 mM HEPES).

Immunoblot Analysis of Bcl-2 Expression

Following homogenization, synaptosome fractions and liver tissues were subjected to protein separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5%) and electroblotted onto a nitrocellulose filter (NC). Rainbow protein markers (Amersham) were used as molecular-weight standard. The NC filter was probed with antihuman Bcl-2 antibodies (Santa Cruz, CA) that were detected with horseradish peroxidase-conjugated antirabbit IgG using the ECL kit (Amersham).

Reactive Oxygen Species (ROS) Production Assay

Synaptosomes were exposed to an oxidative burst by incubation with either H₂O₂ (1 mM, 30 min, 37°C) or with the NO donor 3-morpholininosyd-nimine (SIN-1, 1 mM, 30 min, 37°C). ROS production was measured using a membrane-penetrative diacetate derivative of 2',7'-dichlorofluorescein (DCFH-DA). Upon entering synaptosomes, the diacetate group is cleaved off enzymatically. Both DCFH-DA and DCFH are nonfluorescent fluorescein analogs, however, during oxidative burst they are oxidized to highly fluorescent compounds (DCF; Rosenkranz et al., 1992).

Statistical Analysis

The statistical significance of differences in the clinical severity of EAE following pMOG 35-55-induction between WT and transgenic (NSE-bcl-2) mice groups was evaluated using the Student's *t*-test and contingency table analysis (for correction of small groups) to calculate the χ^2 values with the Stat View 512[®] program (BrainPower Inc).

Results

To study the effects of Bcl-2 overexpression in neurons on the pathogenesis of EAE, we compared the clinical manifestations of the disease in WT (C57BL/6) and transgenic NSE-bcl-2 mice, following disease induction with pMOG 35-55. Two weeks

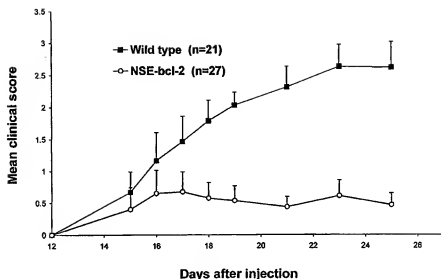


Fig. 1. Induction of EAE in C57BL/6J and NSE-bcl-2 mice with pMOG 35-55. Twenty-one WT (C57BL/6) and 27 transgenic NSE-bcl-2 mice were injected with pMOG 35-55 in CFA supplemented with *M. tuberculosis* (Mt) as described in Materials and Methods. The mean daily clinical score \pm SD is shown for each group of mice (0 = no clinical symptoms; 1 = loss of tail tonicity; 2 = partial hind-limb paralysis; 3 = complete hind-limb paralysis).

after the encephalitogenic challenge, most of the WT mice (18/21) developed severe EAE characterized by complete hind-limb paralysis with mean of score of 2.1 ± 0.14 (Fig. 1). In contrast, the NSE-bcl-2 mice were significantly resistant to MOG-induced EAE. Sixteen out of the 27 (16/27) immunized transgenic mice remained disease-free, ($p < 0.005$ using χ^2 test). The other 11 mice demonstrated mild clinical signs characterized by loss of tail tonicity and some weakness of the hind limbs with mean score of 0.54 ± 0.02 ($p < 0.0001$, using Student's *t*-test). Thus, the disease induced in NSE-bcl-2 mice was markedly reduced both in incidence and clinical severity as compared to that in WT mice.

To further characterize the disease, spinal cords and brains were examined by light microscopy for inflammation and demyelination. Sections of spinal cords from WT mice 45 d after immunization revealed a widespread perivascular lymphohistiocytic inflammatory infiltrate with scattered neutrophils. This was accompanied by severe demyelination and axonal damage (Fig. 2A,B). In contrast, sections of healthy NSE-bcl-2 mice showed only focal perivascular lymphohistiocytic inflammation (Fig. 2D, E), whereas those with mild clinical

signs revealed pathological changes that were not as severe as those seen in the WT mice. Bielshowsky staining of spinal-cord sections from immunized WT mice showed severe axonal damage (Fig. 2C), whereas those from healthy immunized NSE-bcl-2 mice showed minimal axonal damage, only in regions surrounding inflammation only (Fig. 2F). Bielshowsky stained section of the spinal cord from the NSE-bcl-2 mice with mild clinical disease showed a degree of axonal damage intermediate between that seen in the WT mice and that seen in the disease-free NSE-bcl-2 mice (data not shown).

To rule out the possibility that the differences in the clinical manifestations of disease in the NSE-bcl-2 mice were owing to generalized immune dysfunction somehow associated with Bcl-2 overexpression in neurons, the ability of the NSE-bcl-2 mice to mount a T-cell response was compared to that of the WT mice. NSE-bcl-2 and WT mice were immunized with pMOG 35-55 and their recall T-cell proliferative response against the antigen was assessed. As can be seen in Fig. 3, the *in vitro* primary proliferative response of both WT and NSE-bcl-2 mice against pMOG 35-55 was comparable. This indicates that the immune potency of

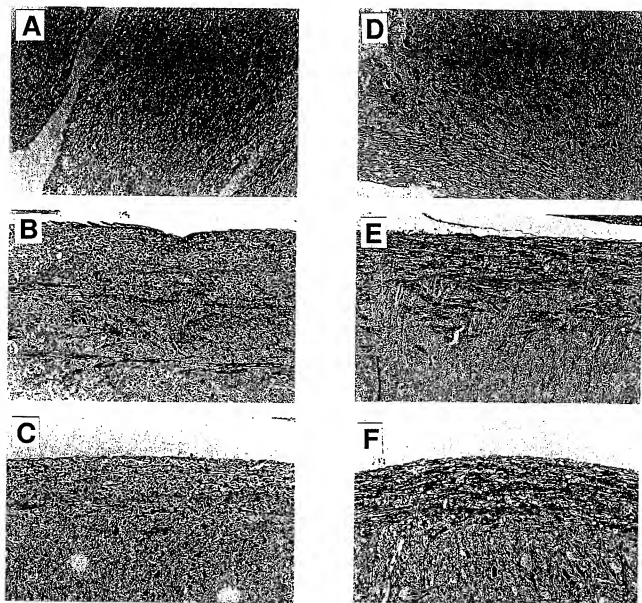


Fig. 2. Histopathology of CNS tissue from WT and NSE-bcl-2 mice with pMOG 35-55-induced EAE. Longitudinal sections of spinal cord from a WT mouse with EAE (A) demonstrating areas of inflammation (H&E). (B) Luxol Fast Blue staining shows loss of myelin, and (C) Bielschowsky staining of the same area indicates axonal loss. Spinal-cord sections from clinically disease-free NSE-bcl-2 mouse stained with H&E show very mild focal inflammation (D). LFB stain shows no loss of myelin (E), and Bielschowsky stain demonstrates an intact axonal population (F). Original magnification of all pictures $\times 100$

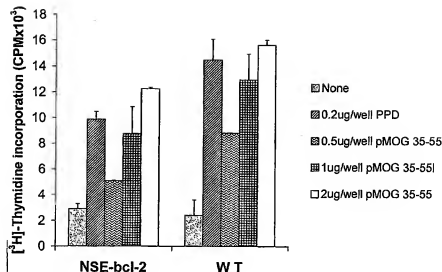


Fig. 3. Proliferative T-cell response against pMOG 35-55 in WT and NSE-bcl-2 mice. Popliteal lymph nodes isolated from mice immunized with the pMOG 35-55 9 d previously, were examined for their primary proliferative T-cell responses to different concentrations (0.5, 1.0, and 2.0 μ g) of peptide. The response to 2.0 μ g of pure protein derivative (PPD) of *M. tuberculosis* was also tested as a positive control of T-cell proliferation. Each histogram represents the mean CPM $\times 10^{-3} \pm$ SEM and of triplicate cultures.

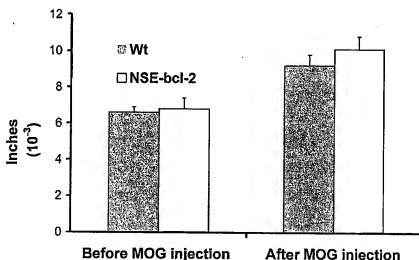


Fig. 4. Delayed-type hypersensitivity (DTH) response against pMOG 35-55 in WT and NSE-bcl-2 mice. DTH response against pMOG 35-55 was assayed by measuring ear thickness following injection with pMOG 35-55 into the right ear of previously sensitized mice. Each histogram represents the mean thickness ($\times 10^{-3}$ inches) \pm SD for each group of mice ($n = 3$).

NSE-bcl-2 mice is similar to that of the WT mice. We also examined the functional capacity of T cells from the two groups of mice to develop a delayed-type hypersensitivity (DTH) response. Figure 4

shows that the DTH response against pMOG 35-55 or against PPD was similar in both WT and NSE-bcl-2 mice. The thickness of the ears as a measure of local inflammation after injection of pMOG

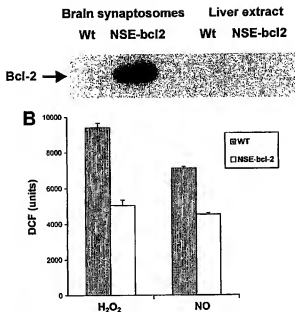


Fig. 5. Free-radical production from purified synaptosomes following an oxidative burst induced with H_2O_2 or NO. (A) The expression of bcl-2 was determined in tissue extracts of brain synaptosomes and liver from WT and NSE-bcl-2 by Western blotting using antihuman bcl-2 antibodies. (B) Purified brain synaptosomes from WT or NSE-bcl-2 mice were exposed to H_2O_2 (1 mM) or the NO-donor, SIN-1 (1 mM) and free-radical production (ROS) was measured by DCFH. Each histogram represents the mean ROS production \pm SD for each group of mice ($n = 3$).

35-55 significantly increased (6.6×10^{-3} inches to 9×10^{-3} inches in WT ($p < 0.01$) and 7×10^{-3} to 9.9×10^{-3} inches in NSE-bcl-2 mice [$p < 0.01$]). Hence, there was no significant difference in the degree of the DTH response between the two mice lines. The thickness in the uninjected ear remained unchanged in all mice (data not shown). These two experiments indicate that there are no differences between the NSE-bcl-2 and WT mice in their capacity to elicit functional encephalitogenic T cells specific for pMOG 35-55.

To further investigate the mechanism(s) underlying reduction in axonal loss and relative resistance to MOG-induced EAE observed in NSE-bcl-2 mice, we assessed the effect of bcl-2 overexpression on the production of ROS, which may lead to axonal damage. We isolated synaptosomes from whole-brain extracts of WT and NSE-bcl-2 mice and confirmed the expression of the human bcl-2 transgene in synaptosomes of transgenic mice by Western-blotting analysis. As shown in Fig. 5A, a 29-kDa band corresponding to human Bcl-2 is

expressed only in synaptosomes but not in the liver of NSE-bcl-2 mice and is not expressed in synaptosomes of WT mice. We then compared free-radical production in synaptosomes from WT and NSE-bcl-2 mice following an oxidative burst by using the DCFH assay (see Methods). Figure 5B shows that ROS production in response to H_2O_2 was significantly higher (9200 vs 4900, $p < 0.01$) in synaptosomes obtained from WT mice, compared with NSE bcl-2 mice. In addition, synaptosomes from NSE-bcl-2 mice produced significantly less ROS (4200 vs 6950, $p < 0.01$) following exposure to induced NO species (Fig. 5B). These results suggest that overexpression of bcl-2 is associated with an increased free-radical scavenger capacity of the synaptosomes.

Discussion

Our study shows that transgenic mice overexpressing bcl-2 in their neurons are highly resistant to MOG-induced EAE. The incidence and clinical

severity of the disease were markedly reduced in these mice compared to WT mice. Furthermore, histological examination of CNS tissues demonstrated reduced levels of demyelination and axonal damage in NSE-bcl-2 compared with WT mice. The resistance to EAE induction is most probably owing to overexpression of bcl-2 in neurons and not to an alteration in the immune response of the transgenic mice. The NSE-bcl-2 transgenic mice were generated using C57BL/6 and therefore differ from WT C57BL/6 only in the overexpression of the human bcl-2 transgene in their neurons (Farlie et al., 1995). Accordingly, the delayed-type hypersensitivity response and in vitro primary T-cell proliferation assays as well as measures of local inflammatory infiltration were similar in WT and transgenic mice. Hence, these data suggest that overexpression of Bcl-2 in neurons can protect against axonal damage and attenuate the clinical manifestations of this experimental CNS demyelinating disease.

In both EAE and MS, the inflammatory process in the CNS plays an important role in demyelination and axonal damage that consequently contributes to disease progression and increasing disability. Such inflammatory changes are associated with local production of highly destructive agents, including NO and ROS, that may contribute to the axonal damage (Vladimirova et al., 1998; Brudin et al., 1999). It has been shown that in rodents with EAE, nonspecific inhibitors of NO-synthetase (NOS) partially ameliorate the disease (Fenyk-Melody et al., 1998). It was suggested that the therapeutic effect of interferon (IFN)- β treatment in MS might be owing, in part, to suppression of pathogenic NO production (Guthikonda et al., 1998). The mechanism by which NO kills cells is not yet understood. However, it appears that NO, together with superoxide, form highly toxic peroxynitrate, which, in turn, may generate additional free radicals that cause harmful sequelae including lipid and protein oxidation (Lipton et al., 1993). Oxidative stress is impotent in the pathogenesis of myelin basic protein (MBP)-induced EAE. For instance, treatment with free-radical scavengers, such as N-acetyl cysteine, inhibited disease development (Lehmann et al., 1994). We have now shown that purified synaptosomes from CNS tissues of NSE-bcl-2 mice, produced significantly less free radicals than those from WT mice when challenged with H_2O_2 and NO.

Bcl-2 plays a major role in the regulation of apoptosis in both the immune and neuronal systems. The exact mechanism by which bcl-2 protects cells from apoptotic death is not yet fully understood. It has been suggested that its antiapoptotic effects are exerted by promoting the function of antioxidants via unknown mechanisms (Frommel and Zarling, 1999). Several in vitro and in vivo studies demonstrated that bcl-2 protects cells from apoptosis induced by endogenous or exogenous oxidants and reduces ROS-induced damage (Hockenbery et al., 1993; Korsmeyer et al., 1995; Hochman et al., 1998; Offen et al., 1998). In addition, it was shown that NO-induced toxicity is prevented by overexpression of bcl-2 (Melkova et al., 1997). Our findings indicate that anti-oxidant and/or anti-apoptotic agents, targeted to the CNS, might prove to be an important treatment modality for attenuating and delaying axonal damage and disease progression.

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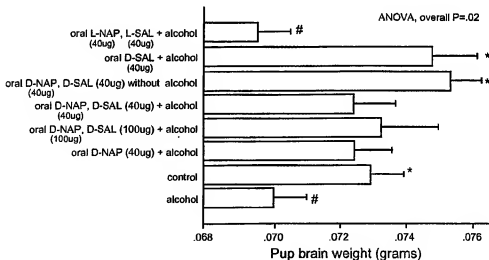
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(54) Title: ORALLY ACTIVE PEPTIDES THAT PREVENT CELL DAMAGE AND DEATH



(57) Abstract: This invention provides an ADNF polypeptide comprising an active core site, the active core site comprising at least one D-amino acid. The invention also provides a pharmaceutical composition comprising an ADNF polypeptide comprising an active core site, the active core site comprising at least one D-amino acid. In particular, the pharmaceutical composition of the invention is orally active. The invention further provides methods for reducing neuronal cell death, methods for reducing oxidative stress, and methods for reducing a condition associated with fetal alcohol syndrome using the ADNF polypeptides and the pharmaceutical compositions of the invention.



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ORALLY ACTIVE PEPTIDES THAT PREVENT CELL DAMAGE AND DEATH

CROSS-REFERENCES TO RELATED APPLICATIONS

- 5 This application claims benefit of the priority of U.S. provisional application number 60/149,956, filed August 18, 1999, the disclosure of which is incorporated herein by reference in its entirety. This application is also related to U.S.S.N. 07/871,973, filed April 22, 1992, now U.S. Patent 5,767,240, issued June 16, 1998; U.S.S.N. 08/342, 297, filed October 17, 1994 (published as WO96/11948);
10 U.S.S.N. 60/037,404, filed February 7, 1997 (published as WO98/35042); U.S.S.N. 09/187,330, filed November 11, 1998; and U.S.S.N. 09/267,511, filed March 12, 1999. All of these applications are incorporated herein by reference.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

- 15 Not applicable.

FIELD OF THE INVENTION

- This invention relates to Activity Dependent Neurotrophic Factor (ADNF)
20 polypeptides comprising at least one D-amino acid within the active core sites of the ADNF polypeptides. The invention also relates to pharmaceutical compositions comprising ADNF polypeptides comprising at least one D-amino acid within the active core sites of the ADNF polypeptides. The invention further relates to methods for reducing neuronal cell death *in vitro* and *in vivo*, methods for treating oxidative stress in a
25 patient, and methods for reducing a condition associated with fetal alcohol syndrome in a subject, all of which methods use the ADNF polypeptides of the invention.

BACKGROUND OF THE INVENTION

- Neuronal cell death has been associated with various clinical conditions
30 and diseases. These conditions and diseases include, for example, neurodegenerative diseases such as Alzheimer's disease, AIDS-related dementia, Huntington's disease, and Parkinson's disease. Neuronal cell death has been also associated with developmental retardation and learning impairments. These diseases and conditions are severely

debilitating and have a lifelong impact on individuals diagnosed with such diseases and conditions.

It has previously been reported that Activity Dependent Neurotrophic Factor (ADNF) polypeptides can be used to prevent or reduce neuronal cell death.

- 5 Activity Dependent Neurotrophic Factor I (ADNF I) polypeptide is secreted by astroglial cells in the presence of vasoactive intestinal peptide (VIP). The ADNF I polypeptide exhibits survival-promoting activity for neurons at surprisingly low, femtomolar concentrations (Brenneman & Gozes, *J. Clin. Invest.* 97:2299-2307 (1996)). Further studies identified peptide fragments of ADNF I that mimic the neurotrophic and
- 10 neuroprotective properties of ADNF I. The shortest peptide (*i.e.*, the active core site) that captured the survival-promoting activity of ADNF I was the peptide SALLRSIPA, designated as ADNF-9 or SAL (Brenneman *et al.*, *J. Pharm. Exp. Therp.* 285:619-627 (1998)). Studies of related molecules to the ADNF I polypeptide resulted in the discovery of Activity Dependent Neuroprotective Protein (called ADNP or ADNF III
- 15 interchangeably). This protein was cloned (Bassan *et al.*, *J. Neurochem.* 72:1283-1293 (1999)) and was found to have an active peptide similar in biological activity to SAL. This peptide (*i.e.*, the active core site) was NAPVSIPQ, designated as NAP.

- ADNF polypeptides have been shown to prevent neuronal cell death both *in vitro* and *in vivo*. For example, ADNF polypeptides have been shown to prevent
- 20 neuronal cell death associated with tetrodotoxin (electrical blockade), the β -amyloid peptide (the Alzheimer's disease neurotoxin), N-methyl-D-aspartate (excitotoxicity), and the human immune deficiency virus envelope protein. In addition, daily injections of ADNF polypeptides to newborn apolipoprotein E-deficient mice accelerated the acquisition of developmental reflexes and prevented short-term memory deficits. *See,*
- 25 *e.g.*, Bassan *et al.*, *J. Neurochem.* 72:1283-1293 (1999). Moreover, pretreatment with ADNF polypeptides has been previously shown to reduce numerous or various conditions associated with fetal alcohol syndrome in a subject. *See*, U.S.S.N. 09/265,511, filed March 12, 1999.

- Although ADNF polypeptides have unlimited potential as neuroprotectants
- 30 and/or therapeutic agents, it would be advantageous to provide additional ADNF polypeptides that have different properties from the known ADNF polypeptides. For example, availability of a number of ADNF polypeptides with different affinities for their receptors would allow targeting specific receptors in different cell types. Furthermore,

additional ADNF polypeptides would aid in designing a drug treatment regime that can be individually tailored for each patient affected by neurodegenerative disorders.

SUMMARY OF THE INVENTION

5 The present invention is based upon a surprising discovery that ADNF polypeptides comprising D-amino acids, which are not present in nature, are also effective for reducing neuronal cell death, for reducing oxidative stress, for reducing condition(s) associated with fetal alcohol syndrome in a subject, and for other conditions. The ADNF polypeptides include ADNF I and ADNF III polypeptides and subsequences
10 thereof which contain their respective active core sites and provide neuroprotective and growth-promoting functions. The ADNF I polypeptides have an active core site comprising the following amino acid sequence: Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala ("SALLRSIPA" or "SAL"; SEQ ID NO:1). The ADNF III polypeptides also have an active core site comprising a few amino acid residues, namely, the following amino acid
15 sequence: Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln ("NAPVSIPQ" or "NAP"; SEQ ID NO:2). ADNF I polypeptides and ADNF III polypeptides comprising all L-amino acids have been previously shown to have remarkable potency and activity for reducing neuronal cell death *in vitro* and *in vivo*, as well as for reducing a condition associated with fetal alcohol syndrome in a subject.

20 As such, in one aspect, the present invention provides an Activity Dependent Neurotrophic Factor I (ADNF I) comprising an active core site having the following amino acid sequence: Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1), wherein the active core site comprises at least one D-amino acid. In one embodiment, the N-terminal and/or the C-terminal amino acids of the active core site of the ADNF I
25 polypeptide are D-amino acids. In another embodiment, the active core site of the ADNF I polypeptide comprises all D-amino acids. In another embodiment, an ADNF I polypeptide is Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1), wherein the ADNF I polypeptide comprises at least one D-amino acid. In another embodiment, the ADNF I polypeptide is Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1), wherein the ADNF
30 I polypeptide comprises all D-amino acids.

 In another aspect, the present invention provides an Activity Dependent Neurotrophic Factor III (ADNF III) comprising an active core site having the following amino acid sequence: Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2), wherein the active core site comprises at least one D-amino acid. In one embodiment, the N-terminal

and/or the C-terminal amino acids of the active core site are D-amino acids. In another embodiment, the active core site of the ADNF III polypeptide comprises all D-amino acids. In another embodiment, the ADNF III polypeptide is Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2), wherein the ADNF III polypeptide comprises at least one D-amino acid. In another embodiment, the ADNF III polypeptide is Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2), wherein the ADNF III polypeptide comprises all D-amino acids.

In yet another aspect, the present invention provides a pharmaceutical composition comprising a pharmaceutically acceptable excipient and an ADNF polypeptide, wherein the ADNF polypeptide is a member selected from the group consisting of: (a) an ADNF I polypeptide comprising an active core site having the following amino acid: Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1); (b) an ADNF III polypeptide comprising an active core site having the following amino acid: Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2); and (c) a mixture of the ADNF I polypeptide or part (a) and the ADNF III polypeptide of part (b); wherein at least one of the ADNF I polypeptide and the ADNF III polypeptide comprises an active core site comprising at least one D-amino acid.

In one embodiment, the pharmaceutical composition comprises an ADNF I polypeptide, wherein the ADNF I polypeptide comprises all D-amino acids. In another embodiment, the pharmaceutical composition comprises an ADNF III polypeptide, wherein the ADNF III polypeptide comprises all D-amino acids. In another embodiment, the pharmaceutical composition comprises an ADNF I polypeptide and an ADNF III polypeptide, wherein the ADNF I polypeptide and the ADNF III polypeptide both comprise all D-amino acids. In another embodiment, the pharmaceutical composition comprises an ADNF I polypeptide and an ADNF III polypeptide, wherein the ADNF I polypeptide comprises all D-amino acids and wherein the ADNF III polypeptide comprises all L-amino acids. In another embodiment, the pharmaceutical composition comprises an ADNF I polypeptide and an ADNF III polypeptide, wherein the ADNF I polypeptide comprises all L-amino acids and wherein the ADNF III polypeptide comprises all D-amino acids.

In yet another aspect, the present invention provides a method for preventing neuronal cell death, the method comprising contacting neuronal cells with at least one of the above described ADNF polypeptides. In one embodiment, the neuronal cell death is in a patient infected with immunodeficiency virus. In another embodiment,

the neuronal cell death is associated with excitotoxicity induced by N-methyl-D-aspartate stimulation. In yet another embodiment, the neuronal cell death is induced by the beta-amyloid peptide in a patient afflicted with Alzheimer's disease. In yet another embodiment, the neuronal cell death is induced by cholinergic blockade in a patient
5 afflicted with Alzheimer's disease, which results in learning impairment.

In yet another aspect, the present invention provides a method for reducing oxidative stress in a patient, the method comprising administering to the patient at least one of the ADNF polypeptides described above in an amount sufficient to treat oxidative stress.

10 In yet another aspect, the present invention provides a method for reducing a condition associated with fetal alcohol syndrome in a subject who is exposed to alcohol *in utero*, the method comprising administering to the subject at least one ADNF polypeptides described above in an amount sufficient to reduce a condition associated with fetal alcohol syndrome.

15

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 compares the survival-promoting activity of D- and L-forms of SALLRSIPA in dissociated cerebral cortical cultures treated with 1 μ M tetrodotoxin, an agent that blocks electrical activity and produces apoptotic neuronal cell death.

20 Treatment duration was for 5 days. Each point is the mean \pm the standard error of 3-4 determinations. Neuronal cell counts were obtained without knowledge of the treatment group.

Figure 2A compares the survival-promoting activity of D- and L-forms of NAPVSIQ in dissociated cerebral cortical cultures treated with 1 μ M tetrodotoxin.

25 Experimental conditions were as described for Figure 1.

Figure 2B illustrates the effect of a mixture of D- and L-amino acid D-NA{L-P}VSIQ on survival promoting activity in cerebral cortical culture co-treated with tetrodotoxin for 5 days. In peptide NAPVSIQ, all of the amino acids were in the D-form, except the third proline residue was in the L-form.

30 Figure 3A compares the survival-promoting activity of combinations of NAPVSIQ and SALLRSIPA in D- and L-forms. Experimental conditions were as described for Figure 1.

Figure 3B compares the survival-promoting activity of combinations of L-NAPVSIPQ + D-SALLRSIPA with D-NAPVSIPQ and L-SALLRSIPA. Experimental conditions were as described for Figure 1.

Figure 4 illustrates that combinations of D-SALLRSIPA + D-NAPVSIPQ
5 can protect against beta amyloid toxicity in PC12 cells.

Figure 5 illustrates that pretreatment with D-NAP, D-SAL, or L-NAP + D-SAL prevents fetal demises. At E18, the number of living and demised embryos was counted and the percentage of demises was calculated. Treatment with alcohol was given on E8, pretreatment with peptides given 30 minutes prior. Comparisons are made to the
10 alcohol group, overall ANOVA $p < 0.001$. Post hoc Fishers tests were performed, with the *groups significantly different than alcohol (all post-hoc $p \leq 0.03$). The sample sizes were control (36), alcohol (41), D-NAP (20 μg) + alcohol (14), D-SAL (20 μg) + alcohol (19), D-SAL (2 μg) + alcohol (8), L-NAP (20 μg) + D-SAL (20 μg) + alcohol (23).

Figure 6A illustrates that pretreatment with L-NAP + D-SAL prevented
15 fetal microcephaly. Fetal brain weights for each pregnant female were obtained at E18. Comparisons are made to the alcohol group, overall ANOVA P value $p = 0.01$. Sample size was the number of litters. The mean from each litter was used for statistical analysis and represents on average 8-10 fetuses. The sample sizes were control (34), alcohol (32), D-NAP (20 μg) + alcohol (13), D-SAL (20 μg) + alcohol (19), D-SAL (2 μg) + alcohol
20 (8), L-NAP (20 μg) + D-SAL (20 μg) + alcohol (23).

Figures 6B illustrates that pretreatment with L-NAP + D-SAL prevented or reduced fetal growth restriction. Fetal weights for each pregnant female were obtained at E18. Comparisons are made to the alcohol group, overall ANOVA P value $p = .04$. Sample size was the number of litters. The mean from each litter was used for statistical
25 analysis and represents on average 8-10 fetuses. The sample sizes were control (34), alcohol (32), D-NAP (20 μg) + alcohol (13), D-SAL (20 μg) + alcohol (19), D-SAL (2 μg) + alcohol (8), L-NAP (20 μg) + D-SAL (20 μg) + alcohol (23).

Figure 7 illustrates that one hour post-treatment with L-NAP and L-SAL prevented fetal death. L-NAP (20 μg) and L-SAL (20 μg) were given at one and three
30 hours after alcohol administration on E8. Comparisons are made to the alcohol group, overall ANOVA $p = 0.001$. Post hoc Fishers tests were performed, with the one-hour and control groups significantly different than alcohol ($p < 0.001$ and $p = 0.04$, respectively).

The sample sizes were control (36), alcohol (41), post one hour treatment (18) and post three hour treatment (14).

Figure 8 illustrates that one and three hour post-treatments with L-NAP and L-SAL prevented fetal microcephaly. L-NAP and L-SAL were given at one and three hours after alcohol administration on E8 (N+S+A). Comparisons are made to the alcohol group, overall ANOVA $P=0.001$. Post hoc Fishers tests were performed, with the one-hour, three hour, and control groups significantly different than alcohol ($p<0.001$, <0.03 and $P<0.008$ respectively). The sample sizes were control (34), alcohol (32), post one hour treatment (17) and post three hour treatment (11).

Figure 9 illustrates that oral treatment with D-NAP and D-SAL prevented fetal death associated with fetal alcohol syndrome. D-NAP and D-SAL were given by gavage immediately after alcohol treatment on E8. Comparisons are made to the alcohol group, overall ANOVA $p=0.004$. Post hoc Fishers tests were performed, with the oral treatment and control groups significantly different than alcohol ($p<0.001$ and ≤ 0.04 respectively). The sample sizes were control (21), alcohol (18), oral D-NAP + D-SAL and alcohol (18).

Figure 10A illustrates the effects of oral administration of ADNF polypeptides on pup brain weight. Pregnant mice were injected with alcohol as a model for fetal alcohol syndrome according to methods of Webster *et al.*, *Neurobehav. Tox.* 2:227-34 (1980). The pregnant mice were injected 25% alcohol at 0.030 ml/g body weight. Peptide was dissolved in phosphate-buffered saline and administered orally by gavage 30 minutes prior to alcohol treatment. The dosage of peptides NAP and SAL administered to each mouse is shown in the figure. Error bars are ± 1 standard errors; * notes significant versus alcohol; and # notes significant versus control.

Figure 10B illustrates the effects of oral administration of ADNF polypeptides on fetal death. Pregnant mice were treated as described above in the description for Figure 10A. The dosage of peptides NAP and SAL administered to each mouse is shown in the figure. Error bars are ± 1 standard errors; * notes significant difference versus alcohol; and # notes significant difference versus control.

Figure 11 illustrates development of cliff avoidance behavior in newborn mice: comparison of peptide drug response in control vs. Apo-E knock-out mice. Animals were treated either by oral application, or subcutaneous injection of D-SAL + D-NAP. Peptides (0.5 mg each) were dissolved in 0.01M acetic acid (30 μ l) and 470 μ l

saline. Further dilutions were performed in saline. For both applications, 0.5 µg of each of the test drugs were delivered; for the oral application (sublingual), in 10 µl saline and for the injection in 20 µl. This protocol was used for the first 4 days of life. From day 5-10, the amount of the peptides and the solution volume was doubled. From day 11-14, the amount of peptide was 2 µg each in 40 µl (oral) and 80 µl (injection). Tests performed daily included cliff avoidance, negative geotaxis, placing and righting behaviors. Both subcutaneous and oral administration of D-NAP and D-SAL were compared.

Figure 12 illustrates development of negative geotaxis behavior in newborn mice: comparison of peptide drug responses in control vs. Apo-E knock-out mice. Treatment paradigm was as described in Figure 11.

Figure 13 illustrates development of placing behavior in newborn mice: comparison of peptide drug responses in control vs. Apo-E knock-out mice. Treatment paradigm was as described in Figure 11.

Figures 14 A and B illustrate the effect of oral administration of D-NAPVSIPQ + D-SALLRSIPA on learning and memory in rats treated with the cholinotoxin AF-64A. Short-term memory processes were examined by performance in the Morris water maze, measuring the time required to find the hidden platform in the second of two daily trials. The platform location and the starting point in which the animal was placed in the water were held constant within each pair of daily trials, but both locations were changed every day. For the first test, both the platform and the animal were situated in a new location with regard to the pool (with the pool being immobile). The experiment was performed as follows: the animal was positioned on the platform for 0.5 minute, then placed in the water. As shown in Figure 14A, the time required to reach the platform (indicative of learning and intact reference memory) was measured (first test). After 0.5 minute on the platform, the animal was placed back in the water (in the previous position) for an additional second test (Figure 14B) and search for the hidden platform (retained in the previous position). The time required to reach the platform in the second trial was recorded, indicative of short-term (working) memory. All measurements were performed using the computerized video-assisted HVS water maze system (HVS Image Ltd. Hampton, UK). Animals were tested for four days to eliminate random memory defective animals. The designated n is the number of animals tested. Each point is the mean + the standard error.

Figure 14C illustrates the effect of oral administration of D-SALLRSIPA alone on learning and memory in rats treated with the cholinotoxin AF-64A.

Figure 15 illustrates comparison of sublingual (oral) and subcutaneous administration of D-SAL + D-NAP in control vs. Apo-E knock-out mice assessed for short-term memory in the Morris swim maze. Improvements of cognitive functions were observed a week after cessation of the 2-week daily D-SAL + D-NAP treatment, *i.e.*, in 21-day-old mice exposed to a 5-day training protocol. The time required to find the hidden platform in the second of two daily trials was measured. The platform location and the starting point in which the animal was placed in the water were held constant within each pair of daily trials, but both locations were changed every day. On the second test of the first trial day, the ApoE-deficient mice were significantly retarded as compared to controls ($P < 0.04$) and improved after oral application of D-SAL + D-NAP, with most of the treated animals finding the platform at a latency of ≤ 20 sec.

Figures 16A and 16B illustrate the first test and second test, respectively, of Morris water maze test results in apolipoprotein E-deficient mice. Experiments were performed following injections of a mixture of D-NAP + D-SAL with an injection protocol and Morris water maze as described in Gozes et al., *J. Pharmacol. Exp. Therap.* 293: 1091-1098 (2000). Results showed significant improvements on day 1 and day 2 (first daily test), and on day three (second daily test)- $P < 0.05$.

DEFINITIONS

The phrase "ADNF polypeptide" refers to one or more activity dependent neurotrophic factors (ADNF) that have an active core site comprising the amino acid sequence of SALLRSIPA or NAPVSIPQ, or conservatively modified variants thereof that have neurotrophic/neuroprotective activity as measured with *in vitro* cortical neuron culture assays described by, *e.g.*, Brenneman et al., *J. Pharmacol. Exp. Therap.* 285:629-627 (1998); Bassan et al., *J. Neurochem.* 72:1283-1293 (1999). An ADNF polypeptide can be an ADNF I polypeptide, an ADNF III polypeptide, their alleles, polymorphic variants, or interspecies homolog, or any subsequences thereof, such as NAP and SAL, that exhibit neuroprotective/neurotrophic action on, *e.g.*, neurons originating in the central nervous system either *in vitro* or *in vivo*. An "ADNF polypeptide" can also refer to a mixture of ADNF I polypeptide and ADNF III polypeptide.

The term "ADNF I" refers to an activity dependent neurotrophic factor polypeptide having a molecular weight of about 14,000 Daltons with a pI of 8.3 ± 0.25 . As described above, ADNF I polypeptides have an active core site comprising an amino acid sequence of Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (also referred to as

5 "SALLRSIPA," "SAL," or "ADNF I-9"; SEQ ID NO:1). See, Brenneman *et al.*, *J. Clin. Invest.* 97:2299-2307 (1996), Glazner *et al.*, *Anat. Embryol.* 200:65-71 (1999), Brenneman *et al.*, *J. Pharm. Exp. Ther.* 285:619-27 (1998), Gozes & Brenneman, *J. Mol. Neurosci.* 7:235-244 (1996), and Gozes *et al.*, *Dev. Brain Res.* 99:167-175 (1997), all of which are herein incorporated by reference. Unless indicated as otherwise, "SAL" refers

10 to a peptide having an amino acid sequence of Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1), not a peptide having an amino acid sequence of Ser-Ala-Leu.

The terms "ADNF III" and "ADNP" refer to an activity dependent neurotrophic factor polypeptide having a predicted molecular weight of about 95 kDa (about 828 amino acid residues) and a pI of about 5.99. As described above, ADNF III

15 polypeptides have an active core site comprising an amino acid sequence of Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (also referred to as "NAPVSIPQ," "NAP," or "ADNF III-8"; SEQ ID NO:2). See, Bassan *et al.*, *J. Neurochem.* 72:1283-1293 (1999), incorporated herein by reference. Unless indicated as otherwise, "NAP" refers to a peptide having an amino acid sequence of Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2), not a peptide having

20 an amino acid sequence of Asn-Ala-Pro.

The phrase "reducing neuronal cell death" refers to reduction, including prevention, of neuronal cell death. Reduction is a change of a parameter by about 10% to about 100%, preferably at least about 50%, and more preferably at least about 80% compared to that of the control (*e.g.*, without treatment with, *e.g.*, ADNF polypeptides).

25 The reduction of neuronal cell death can be measured by any methods known in the art. For example, ADNF polypeptides that reduce neuronal cell death can be screened using the various methods described in U.S.S.N. 60/037,404, filed February 27, 1997 (published as WO98/35042) and U.S.S.N. 09/187,330, filed November 6, 1998, both of which are incorporated herein by reference.

30 The phrase "oxidative stress" in cells or tissues refers to enhanced generation of free radicals or reactive oxygen species (ROS) (such as α -hydroxy ethyl radical, superoxide radical, hydroxy radical, peroxy radical, and hydrogen peroxide) and/or a depletion in antioxidant defense system causing an imbalance between

prooxidants and antioxidants. Enzymatic antioxidant system includes, *e.g.*, superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, and nonenzymatic antioxidants include, *e.g.*, reduced glutathione, vitamin A, C, and E. *See*, Schlorff *et al.*, *Alcohol* 17:97-105 (1999).

- 5 The phrase "reducing oxidative stress" refers to reduction, including prevention, of oxidative stress in cells and tissues. Reduction is a change of a parameter by about 10% to about 100%, preferably at least about 50%, and more preferably at least about 80% compared to that of the control (*e.g.*, without treatment with, *e.g.*, ADNF polypeptides). The reduction in oxidative stress can be measured by any methods known
- 10 in the art. For example, ADNF polypeptides that reduce oxidative stress can be screened by using primary neurons treated with FeSO_4 *in vitro* as described *infra*. Also, ADNF polypeptides that reduce oxidative stress can be screened using animals that ingested ethanol which is known to cause oxidative stress in cells and tissues. For example, the effects of ADNF polypeptides on lipid peroxidation in plasma and/or antioxidant system
- 15 of rats that ingested ethanol can be used. *See, e.g.*, Schlorff *et al.*, *Alcohol* 17:97-105 (1999).

- The phrases "fetal alcohol syndrome" and "fetal alcohol effects" relate to various physical and mental conditions of an embryo, a fetus, or a subject who is exposed to alcohol *in utero* (*e.g.*, whose mother consumed alcohol during pregnancy) in an amount
- 20 sufficient to initiate the development of these conditions or to cause these conditions in the absence of prevention treatment, *e.g.*, treatment with ADNF polypeptides. Some of these conditions include, but are not limited to, the following:
- skeletal deformities: deformed ribs and sternum; curved spine; hip dislocations; bent, fused, webbed, or missing fingers or toes; limited movement of joints;
- 25 small head; facial abnormalities: small eye openings; skin webbing between eyes and base of nose; drooping eyelids; nearsightedness; failure of eyes to move in same direction; short upturned nose; sunken nasal bridge; flat or absent groove between nose and upper lip; thin upper lip; opening in roof of mouth; small jaw; low-set or poorly formed ears; organ deformities: heart defects; heart murmurs; genital malformations;
- 30 kidney and urinary defects; central nervous system handicaps: small brain; faulty arrangement of brain cells and connective tissue; mental retardation - usually mild to moderate, but occasionally severe; learning disabilities; short attention span; irritability in infancy; hyperactivity in childhood; poor body, hand, and finger coordination (*see, e.g.*, www.well.com/user/woa/fsfas.htm); and other abnormalities: brain weight reduction,

body weight reduction, a higher rate of death *in utero*, and a decrease in the level of VIP (e.g., VIP mRNA).

The phrase "reducing a condition associated with fetal alcohol syndrome" refers to reduction, including prevention, of parameters associated with fetal alcohol syndrome. Reduction is a change of a parameter by about 10% to about 100%, preferably at least about 50%, and more preferably at least about 80% compared to that of the control (e.g., exposed to alcohol *in utero* without any treatment, e.g., treatment with ADNF polypeptides). The parameters can be any physical or mental condition listed above. For example, they can be: (1) the percentage of fetus death, (2) fetal weights and fetal brain weights, (3) the level of VIP (e.g., VIP mRNA) in embryos, (4) learning and/or memory, and (5) the glutathione level.

The phrase "a subject with fetal alcohol syndrome" relates to an embryo, a fetus, or a subject, in particular a human, who is exposed to alcohol *in utero* and who has fetal alcohol syndrome or who is at risk or in danger of developing, due to maternal alcohol consumption, any of the conditions related to fetal alcohol syndrome, such as the effects described above.

The term "memory" includes all medical classifications of memory, e.g., sensory, immediate, recent and remote, as well as terms used in psychology, such as reference memory, which refers to information gained from previous experience, either recent or remote (see, e.g., *Harrison's Principles of Internal Medicine*, volume 1, pp. 142-150 (Fauci *et al.*, eds., 1988)).

Various parameters can be measured to determine if an ADNF polypeptide or a mixture of ADNF polypeptides improves performance of a subject (e.g., learning and memory). For example, the degree of learning deficits can be compared between the control (e.g., untreated with ADNF polypeptides) and a group pretreated with ADNF polypeptides. The phrase "improving learning and memory" refers to an improvement or enhancement of at least one parameter that indicates learning and memory. Improvement or enhancement is change of a parameter by at least 10%, optionally at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 150%, at least about 200%, *etc.* The improvement of learning and memory can be measured by any methods known in the art. For example, ADNF polypeptides that improve learning and memory can be screened using Morris water maze (see, e.g., the

materials and methods section). *See, also*, Gozes et al., *Proc. Natl. Acad. Sci. USA* 93:427-432 (1996); Gozes et al., *J. Pharmacol. Exp. Therap.* 293: 1091-1098 (2000).

The term "contacting" is used herein interchangeably with the following: combined with, added to, mixed with, passed over, incubated with, flowed over, etc.

- 5 Moreover, the ADNF polypeptides of the present invention can be "administered" by any conventional method such as, for example, parenteral, oral, topical, and inhalation routes. In preferred embodiments, oral administration is employed. In the context of methods related to fetal alcohol syndrome, ADNF polypeptides can be administered directly to an embryo, a fetus, or a subject *in utero* or to the subject *in utero* indirectly, by administering
10 the polypeptide to the mother by any other methods described herein.

- "An amount sufficient" or "an effective amount" is that amount of a given ADNF polypeptide that reduces neuronal cell death or reduces fetal alcohol syndrome or oxidative stress as described herein. For example, in the context of neuronal death, "an amount sufficient" or "an effective amount" is that amount of a given ADNF polypeptide
15 that reduces neuronal cell death in the assays of, e.g., Hill et al., *Brain Res.* 603:222-233 (1993); Brenneman et al., *Nature* 335:639-642 (1988); or Brenneman et al., *Dev. Brain Res.* 51:63-68 (1990); Forsythe & Westbrook, *J. Physiol. Lond.* 396:515-533 (1988). In the context of reducing oxidative stress, "an amount sufficient" or "an effective amount" is that amount of ADNF polypeptide that reduces or prevents, e.g., changes in lipid
20 peroxidation in plasma or changes in antioxidant system in accordance with the assays described in Schlorff et al., *Alcohol* 17:97-105 (1999). In the context of reducing fetal alcohol syndrome, "an amount sufficient" or "an effective amount" is that amount of a given ADNF polypeptide that reduces or prevents, for example, (1) the percentage of fetus death, (2) a reduction in fetal weights and fetal brain weights, or (3) a reduction in
25 the level of VIP mRNA in embryos. The dosing range can vary depending on the ADNF polypeptide used, the route of administration and the potency of the particular ADNF polypeptide, but can readily be determined using the foregoing assays.

- The term "biologically active" refers to a peptide sequence that will interact with naturally occurring biological molecules to either activate or inhibit the
30 function of those molecules *in vitro* or *in vivo*. The term "biologically active" is most commonly used herein to refer to ADNF polypeptides or subsequences thereof that exhibit neuroprotective/neurotrophic action on neurons originating in the central nervous system either *in vitro* or *in vivo*. The neuroprotective/neurotrophic action of ADNF

polypeptides can be tested using, *e.g.*, cerebral cortical cultures treated with a neurotoxin (see, Gozes *et al.*, *Proc. Nat'l. Acad. Sci. USA* 93:427-432 (1996)).

The terms "isolated," "purified," or "biologically pure" refer to material that is substantially or essentially free from components which normally accompany it as found in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated ADNF nucleic acid is separated from open reading frames that flank the ADNF gene and encode proteins other than ADNF. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

"Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

The term "amino acid" refers to naturally occurring amino acids in L-form and their enantiomers in D-form, amino acid analogs, and amino acid mimetics. The two-mirror-image forms (enantiomers) of amino acids are called the L-isomer and the D-isomer, where L refers to levorotatory (left rotation of the plane of polarization of light) and D refers to dextrorotatory (right rotation of the plane of polarization). The term "amino acid" also includes amino acids that are later modified, *e.g.*, hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refer to synthetic amino

acids that have the same basic chemical structure as naturally occurring amino acids in L-form or their enantiomers in D-form, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group (*e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium). Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refer to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid. Amino acid analogs and amino acids mimetics can also be in L-form or in D-form.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refer to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule.

Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not
10 exclude polymorphic variants, interspecies homologs, and alleles of the invention.

The following groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Glycine (G);
 - 2) Serine (S), Threonine (T);
 - 15 3) Aspartic acid (D), Glutamic acid (E);
 - 4) Asparagine (N), Glutamine (Q);
 - 5) Cysteine (C), Methionine (M);
 - 6) Arginine (R), Lysine (K), Histidine (H);
 - 7) Isoleucine (I), Leucine (L), Valine (V); and
 - 20 8) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).
- (see, e.g., Creighton, *Proteins* (1984)).

The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is a naturally occurring
25 amino acid in L-form or their enantiomers in D-form, an analog or mimetic of amino acids in L-form or D-form, or combinations thereof.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, 70% identity, optionally 75%, 80%, 85%, 90%, or 95% identity over a
30 specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Such sequences are then said to be "substantially identical." This definition also refers to the

compliment of a test sequence. Optionally, the identity exists over a region that is at least about 50 amino acids or nucleotides in length, or more preferably over a region that is 75-100 amino acids or nucleotides in length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, e.g., *Current Protocols in Molecular Biology* (Ausubel et al., eds. 1995 supplement)).

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences,

producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. Using PILEUP, a reference sequence is compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, *e.g.*, version 7.0 (Devereaux *et al.*, *Nuc. Acids Res.* 12:387-395 (1984).

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as

defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity
5 between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest
10 sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is
15 immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to
20 each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

A further indication that two polynucleotides are substantially identical is if the reference sequence, amplified by a pair of oligonucleotide primers or a pool of
25 degenerate primers that encode a conserved amino acid sequence, can then be used as a probe under stringent hybridization conditions to isolate the test sequence from a cDNA or genomic library, or to identify the test sequence in, e.g., a northern or Southern blot. Alternatively, another indication that the sequences are substantially identical is if the same set of PCR primers can be used to amplify both sequences.

30 The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (e.g., total cellular or library DNA or RNA).

The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10 °C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with a wash in 0.2x SSC, and 0.1% SDS at 65°C.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

I. INTRODUCTION

The chirality (left or right handedness) of a peptide pertains to the tetrahedral array of four different groups about the α -carbon atom of the constituent amino acids that confers optical activity. The two-mirror-image forms (enantiomers) of amino acids are called the L-isomer and the D-isomer, where L refers to levorotatory (left rotation of the plane of polarization of light) and D refers to dextrorotatory (right rotation of the plane of polarization). Only L-amino acids are constituents of naturally occurring proteins. Classical receptor pharmacology teaches that membrane receptors readily discriminate between L- and D-agonists and antagonists. Receptor activation is mediated through a stereoselective preference for agents in the naturally occurring L-isomer form.

Because ADNF I and ADNF III polypeptides are neurotrophic factors, it was predicted that ADNF I and ADNF III polypeptides comprising D-amino acids would not be able to activate their respective stereoselective membrane receptors. Surprisingly, it was found that ADNF I and ADNF III polypeptides comprising D-amino acids were bioactive. In fact, all D- and all L-amino acid forms of the active core site peptide from ADNF I polypeptides, *i.e.*, SALLRSIPA (SAL), were virtually identical in neuronal survival activity *in vitro*. Similarly, all D- and all L-amino acid forms of the active core site peptide from ADNF III polypeptides, *i.e.*, NAPVSIPQ (NAP), were virtually identical in neuronal survival activity *in vitro*. It is very uncommon that all D-amino acid peptides are active, and even more uncommon that the D- and L-isomers of a given peptide are equally active.

A few examples of peptides with similar actions in D- vs. L-forms have been reported. A well-known example is beta amyloid. It was shown that bioactivity of D-isomers of beta amyloid 1-42 is identical to that observed with the L-form of the peptide (Cribbs *et al.*, *J. Biol. Chem.* 272:7431-7436 (1997)). Another example is the immunosuppressive effects of D- and L-peptides derived from the HLA class I heavy chain (Woo *et al.*, *Transplantation* 64:1460-1467 (1997)). Although these examples illustrate that bioactivity of peptides can be non-stereoselective, this phenomenon is very rare. This is because biological macromolecules are made up of monomer molecules of uniform chirality (Mason, *Chirality* 3:223 (1990)) and the biochemical interactions of biological macromolecules are inherently chiral. In fact, for neurotrophic agents, there is

no known example that exhibits non-chiral properties. Thus, it is surprising that ADNF polypeptides of the present invention provide neuroprotection through a non-chiral mechanism.

The fact that ADNF polypeptides comprising D-amino acids are bioactive allows these polypeptides to be administered orally. Compared to L-isomers, D-isomers of peptides have increased stability in the gastrointestinal tract and can be absorbed without change (He *et al.*, *J. Pharmaceutical Sci.* 87:626-633 (1998)). For example, in He *et al.*, bioavailability (as measured by the appearance of unchanged labeled D-peptides in the urine after 24-48 hours) was estimated at 13% with compounds of molecular mass of 900 Daltons, the approximate size of the active core sites of ADNF I and ADNF III polypeptides. ADNF polypeptides comprising D-amino acids provide a longer bioavailability, and thus can be formulated for oral administration.

As such, the present invention provides for the first time, *inter alia*, ADNF polypeptides comprising at least one D-amino acid within their active core sites, preferably at the N-terminus and/or the C-terminus of the active core sites. In a presently preferred embodiment, the invention provides ADNF polypeptides comprising all D-amino acids. The invention also provides a pharmaceutical composition comprising a pharmaceutically acceptable excipient and an ADNF I polypeptide, an ADNF III polypeptide, or a mixture thereof, wherein at least one of the ADNF I polypeptide or the ADNF III polypeptide comprises at least one D-amino acid within its active core site. In particular, the invention provides an orally active pharmaceutical composition comprising an ADNF polypeptide comprising at least one D-amino acid within its active core site. The ADNF polypeptides and the pharmaceutical compositions of the present invention can be used, *inter alia*, in methods for reducing neuronal cell death, for reducing oxidative stress in a patient, and for reducing a condition associated with fetal alcohol syndrome.

II. ADNF POLYPEPTIDES COMPRISING D-AMINO ACIDS AND METHODS OF MAKING THE POLYPEPTIDES

In one aspect, the present invention provides an ADNF polypeptide comprising at least one D-amino acid within its active core site, preferably at the N-terminus and/or the C-terminus of the active core site. Since D-enantiomers of polypeptides are enzymatically more stable than their L-enantiomers, an ADNF polypeptide comprising D-amino acids provides a longer bioavailability compared to its

counterpart comprising L-amino acids. In particular, the ADNF polypeptides comprising D-amino acids are stable in the gastrointestinal tract and can be absorbed without cleavage in the human body. Therefore, the ADNF polypeptides of the present invention are particularly useful as an oral agent.

- 5 In one embodiment, the ADNF I polypeptide comprises an active core site having the following amino acid sequence: Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1), wherein the active core site comprises at least one D-amino acid. In another embodiment, both the N-terminal and/or the C-terminal amino acids of the active core site of the ADNF I polypeptide are D-amino acids. In yet another embodiment, the active
- 10 core site of the ADNF I polypeptide comprises D-amino acids at locations other than at the N-terminus and/or the C-terminus of the active core site. For example, any amino acids within the active core site can be a D-amino acid. In other words, any one or any combinations of serine, alanine, leucine, leucine, arginine, serine, isoleucine, proline, and alanine within the active core site of the ADNF I polypeptides can be a D-amino acid.
- 15 For instance, every other amino acid within the active core site of the ADNF I polypeptide can be a D-amino acid. In a preferred embodiment, the active core site of the ADNF I polypeptide comprises all D-amino acids.

- In yet another embodiment, the ADNF I polypeptide can comprise additional amino acids at the N-terminus and/or at the C-terminus of the active core site.
- 20 For example, the ADNF I polypeptide can comprise up to 40 amino acids at the N-terminus and/or the C-terminus of the active core site. In another example, the ADNF I polypeptide can comprise up to 20 amino acids at the N-terminus and/or the C-terminus of the active core site. In yet another example, the ADNF I polypeptide can comprise up to 10 amino acids at the N-terminus and/or the C-terminus of the active core site. In these
- 25 embodiments, preferably the N-terminal amino acid and/or the C-terminal amino acid of the ADNF I polypeptide are D-amino acids. Any one of the additional amino acids or all of the additional amino acids can be D-amino acids. In a preferred embodiment, the ADNF I polypeptide does not comprise any additional amino acids and has an amino acid sequence of Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1), wherein the ADNF I
- 30 polypeptide comprises all D-amino acids.

In another embodiment, the ADNF III polypeptide comprises an active core site having the following amino acid sequence: Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2), wherein the active core site comprises at least one D-amino acid. In another embodiment, both the N-terminal and/or C-terminal amino acids of the active

core site of the ADNF III polypeptide are D-amino acids. In yet another embodiment, the active-core site of the ADNF III polypeptide comprises D-amino acids at locations other than at the N- or C-terminus of the active core site. For example, any amino acids within the active core site can be a D-amino acid. In other words, any one or any combination of asparagine, alanine, proline, valine, serine, isoleucine, proline, and glutamine within the active core site of the ADNF III polypeptides can be a D-amino acid. For instance, every other amino acid within the active core site of the ADNF III polypeptide can be a D-amino acid. In a preferred embodiment, the active core site of the ADNF III polypeptide comprises all D-amino acids.

In yet another embodiment, the ADNF III polypeptide can comprise additional amino acids at the N-terminus and/or at the C-terminus of the active core site. For example, the ADNF III polypeptide can comprise up to 40 amino acids at the N-terminus and/or the C-terminus of the active core site. In another example, the ADNF III polypeptide can comprise up to 20 amino acids at the N-terminus and/or the C-terminus of the active core site. In yet another example, the ADNF III polypeptide can comprise up to 10 amino acids at the N-terminus and/or the C-terminus of the active core site. In these embodiments, preferably the N-terminal amino acid and/or the C-terminal amino acid of the ADNF I polypeptide are D-amino acids. Any one of the additional amino acids or all of the additional amino acids can be D-amino acids. In a preferred embodiment, the ADNF III polypeptide does not comprise any additional amino acids and has an amino acid sequence of Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2), wherein the ADNF III polypeptide comprises all D-amino acids.

In a preferred embodiment, the ADNF I polypeptide comprises an amino acid sequence having the following formula: $(R^1)_x$ -Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala- $(R^2)_y$ (SEQ ID NO:3), wherein the active core site comprises at least one D-amino acid. In another preferred embodiment, the ADNF III polypeptide comprises an amino acid sequence having the following formula: $(R^3)_w$ -Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln- $(R^4)_z$ (SEQ ID NO:4), wherein the active core site comprises at least one D-amino acid. In these preferred embodiments, the previous discussion pertaining to the location and the number of D-amino acids within the active core sites of the ADNF I and ADNF III polypeptides is fully applicable, and thus, will not be repeated with respect to these particular embodiments of the invention.

In the above formula, each of R^1 , R^2 , R^3 , and R^4 , if present, is an amino acid sequence comprising from 1 to about 40 amino acids wherein each amino acid in the

amino acid sequence is independently selected. The term "independently selected" is used herein to indicate that the amino acids making up, for example, the amino acid sequence R^1 may be identical or different (e.g., all of the amino acids in the amino acid sequence may be threonine, *etc.*). This discussion pertaining to R^1 is fully applicable to

5 R^2 , R^3 , and R^4 . Moreover, any one or any combinations of the amino acids making up the amino acid sequence R^1 can be a D-amino acid or an L-amino acid. In one embodiment, the N-terminal amino acid of R^1 is a D-amino acid and/or the C-terminal amino acid of R^2 is a D-amino acid. In another embodiment, the N-terminal amino acid of R^3 is a D-amino acid and/or the C-terminal amino acid of R^4 is a D-amino acid. In another embodiment,

10 each of R^1 , R^2 , R^3 , and R^4 comprises all D-amino acids.

Within the above formula for the ADNF I polypeptide, x and y are independently selected and are equal to zero or one. The term independently selected is used herein to indicate that x and y may be identical or different. For example, x and y may both be zero or, alternatively, x and y may both be one. In addition, x may be zero

15 and y may be one or, alternatively, x may be one and y may be zero. Moreover, if x and y are both one, the amino acid sequences R^1 and R^2 may be the same or different. As such, the amino acid sequences R^1 and R^2 are independently selected. If R^1 and R^2 are the same, they are identical in terms of both chain length and amino acid composition. For example, both R^1 and R^2 may be Val-Leu-Gly-Gly-Gly (SEQ ID NO:5). If R^1 and R^2 are

20 different, they can differ from one another in terms of chain length and/or amino acid composition and/or order of amino acids in the amino acids sequences. For example, R^1 may be Val-Leu-Gly-Gly-Gly (SEQ ID NO:5), whereas R^2 may be Val-Leu-Gly-Gly (SEQ ID NO:9). Alternatively, R^1 may be Val-Leu-Gly-Gly-Gly (SEQ ID NO:5), whereas R^2 may be Val-Leu-Gly-Gly-Val (SEQ ID NO:13). Alternatively, R^1 may be

25 Val-Leu-Gly-Gly-Gly (SEQ ID NO:5), whereas R^2 may be Gly-Val-Leu-Gly-Gly (SEQ ID NO:11).

Similarly, w and z are independently selected and are equal to zero or one within the above formula for the ADNF III polypeptide. The term independently selected is used herein to indicate that w and z may be identical or different. For example, w and z

30 may both be zero or, alternatively, w and z may both be one. In addition, w may be zero and z may be one or, alternatively, w may be one and z may be zero. Moreover, if w and z are both one, the amino acid sequences R^3 and R^4 may be the same or different. As such, the amino acid sequences R^3 and R^4 are independently selected. If R^3 and R^4 are the same, they are identical in terms of both chain length and amino acid composition.

For example, both R³ and R⁴ may be Leu-Gly-Leu-Gly-Gly (SEQ ID NO:7). If R³ and R⁴ are different, they can differ from one another in terms of chain length and/or amino acid composition and/or order of amino acids in the amino acids sequences. For example, R³ may be Leu-Gly-Leu-Gly-Gly (SEQ ID NO:7), whereas R⁴ may be Leu-Gly-Leu-Gly (SEQ ID NO:12). Alternatively, R³ may be Leu-Gly-Leu-Gly-Gly (SEQ ID NO:7), whereas R⁴ may be Leu-Gly-Leu-Gly-Leu (SEQ ID NO:13).

Within the scope, certain ADNF I and ADNF III polypeptides are preferred, namely those in which x, y, w, and z are all zero (*i.e.*, SALLRSIPA (SEQ ID NO:1) and NAPVSIQ (SEQ ID NO:2), respectively). Equally preferred are ADNF I polypeptides in which x is one; R¹ is Val-Leu-Gly-Gly-Gly (SEQ ID NO:5); and y is zero. Also equally preferred are ADNF I polypeptides in which x is one; R¹ is Val-Glu-Gly-Gly-Ile-Val-Leu-Gly-Gly-Gly (SEQ ID NO:6); and y is zero. Also equally preferred are ADNF III polypeptides in which w is one; R³ is Gly-Gly; and z is zero. Also equally preferred are ADNF III polypeptides in which w is one; R³ is Leu-Gly-Gly; z is one; and R⁴ is Gln-Ser. Also equally preferred are ADNF III polypeptides in which w is one; R³ is Leu-Gly-Leu-Gly-Gly- (SEQ ID NO:7); z is one; and R⁴ is Gln-Ser. Also equally preferred are ADNF III polypeptides in which w is one; R³ is Ser-Val-Arg-Leu-Gly-Leu-Gly-Gly (SEQ ID NO:8); z is one; and R⁴ is Gln-Ser. Additional amino acids can be added to both the N-terminus and the C-terminus of these active core sites (SALLRSIPA or NAPVSIQ) without loss of biological activity as evidenced by the fact that the intact ADNF I or ADNF III growth factors exhibit extraordinary biological activity. *See*, U.S.S.N. 08/324,297, filed October 17, 1994 (also published as WO96/11948) for the description of ADNF I polypeptides; and U.S.S.N. 60/037,404 filed February 27, 1997 and U.S.S.N. 60/059,621 filed, September 23, 1997 (also published as WO98/35042) for the description of ADNF III polypeptides, all of which are incorporated herein by reference.

The ADNF polypeptides comprising at least one D-amino acid within the active core sites of the ADNF polypeptides can be prepared via a wide variety of well-known techniques. Polypeptides of relatively short size are typically synthesized in solution or on a solid support in accordance with conventional techniques (*see, e.g.*, Merrifield, *Am. Chem. Soc.* 85:2149-2154 (1963)). Various automatic synthesizers and sequencers are commercially available and can be used in accordance with known protocols (*see, e.g.*, Stewart & Young, *Solid Phase Peptide Synthesis* (2nd ed. 1984)). Solid phase synthesis in which the C-terminal amino acid of the sequence is attached to

an insoluble support followed by sequential addition of the remaining amino acids in the sequence is the preferred method for the chemical synthesis of the polypeptides of this invention. Using solid phase synthesis methods, one or more D-amino acids can be inserted, instead of L-amino acids, into an ADNF polypeptide at any desired location(s).

- 5 Techniques for solid phase synthesis are described by Barany & Merrifield, *Solid-Phase Peptide Synthesis*; pp. 3-284 in *The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part A.*; Merrifield et al., *J. Am. Chem. Soc.* 85:2149-2156 (1963); and Stewart et al., *Solid Phase Peptide Synthesis* (2nd ed. 1984).

- Alternatively, ADNF polypeptides comprising at least one D-amino acid
10 within their active core sites can be synthesized using both recombinant DNA methods and chemical synthesis. For example, fragments of an ADNF polypeptide comprising D-amino acids can be chemically synthesized using solid phase synthesis methods described above, and fragments of an ADNF polypeptide comprising L-amino acids can be produced recombinantly. That is, expression vectors containing a nucleic acid encoding a
15 fragment of an ADNF polypeptide can be introduced into host cells, and then the expressed ADNF polypeptide fragments can be purified. These ADNF polypeptide fragments comprising D-amino acids and ADNF polypeptide fragments comprising L-amino acids can then be chemically linked to one another.

- After chemical synthesis, biological expression or purification, the
20 polypeptide(s) may possess a conformation substantially different than the native conformations of the constituent polypeptides. In this case, it is helpful to denature and reduce the polypeptide and then to cause the polypeptide to re-fold into the preferred conformation. Methods of reducing and denaturing polypeptides and inducing re-folding are well known to those of skill in the art (see Debinski et al., *J. Biol. Chem.* 268:14065-
25 14070 (1993); Kreitman & Pastan, *Bioconjug. Chem.* 4:581-585 (1993); and Buchner et al., *Anal. Biochem.* 205:263-270 (1992)). Debinski et al., for example, describe the denaturation and reduction of inclusion body polypeptides in guanidine-DTE. The polypeptide is then refolded in a redox buffer containing oxidized glutathione and L-arginine.

- 30 One of skill will appreciate that many conservative variations of the ADNF polypeptide sequences provided herein yield functionally identical products. For example, due to the degeneracy of the genetic code, "silent substitutions" (i.e., substitutions of a nucleic acid sequence that do not result in an alteration in an encoded polypeptide) are an implied feature of every nucleic acid sequence that encodes an amino

acid. Similarly, "conservative amino acid substitutions," in one or a few amino acids in an amino acid sequence are substituted with different amino acids with highly similar properties (*see* the definitions section, *supra*), are also readily identified as being highly similar to a disclosed amino acid sequence, or to a disclosed nucleic acid sequence that
5 encodes an amino acid. Such conservatively substituted variations of each explicitly listed nucleic acid and amino acid sequences are a feature of the present invention.

Moreover, one of skill will recognize that other modifications can also be made to the ADNF polypeptides comprising at least one D-amino acid without diminishing their biological activity. For example, modifications can be made to avoid
10 cleavage by enzymes in the stomach or intestines. In another example, modifications can be made to aid the purification process.

It will be readily apparent to those of ordinary skill in the art that the biologically active ADNF polypeptides of the present invention can readily be screened for neuroprotective/neurotrophic activity using a number of methods known in the art.
15 For example, a cerebral cortical cell culture assay can be used. In cerebral cortical cell culture assays, cerebral cortical cell cultures are prepared using the techniques described by Forsythe & Westbrook, *J. Physiol. Lond.* 396:515-533 (1988) with the following modifications. Cerebral cortex are used instead of hippocampus, and newborn rats are used instead of E16 mice. After nine days growth *in vitro*, the cultures are given a
20 complete change of medium and treated with the ADNF polypeptide of interest (dissolved in phosphate buffered saline) for an additional five days. To terminate, the cells are fixed for immunocytochemistry and neurons identified with antibodies against NSE (*i.e.*, neuron specific enolase, a neuronal specific marker). Cell counts are performed on 30 fields, with total area of about 15 mm². Neurons are counted without knowledge of
25 treatment. Control counts not treated with any drugs should run for purposes of comparison. Furthermore, assays described by, *e.g.*, Hill *et al.*, *Brain Res.* 603:222-233 (1993).

Using these assays, one of ordinary skill in the art can readily prepare a large number of ADNF polypeptides in accordance with the teachings of the present
30 invention and, in turn, screen them using the foregoing assay to find ADNF polypeptides, in addition to those set forth herein, which possess the neuroprotective/neurotrophic activity of the intact ADNF growth factor. For instance, using ADNF III-8 (*i.e.*, Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln; SEQ ID NO:2) as a starting point, one can systematically add, for example, Gly-, Gly-Gly-, Leu-Gly-Gly- to the N-terminus of ADNF III-8 and, in turn,

screen each of these ADNF III polypeptides in the foregoing assay to determine whether they possess neuroprotective/ neurotrophic activity. In doing so, it will be found that additional amino acids can be added to both the N-terminus and the C-terminus of the newly discovered active core site, *i.e.*, Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2),
5 without loss of biological activity as evidenced by the fact that the intact ADNF III growth factor exhibits extraordinary biological activity. It will be readily apparent to those of skill in the art that this discussion also applies to ADNF I polypeptides.

III. PHARMACEUTICAL COMPOSITIONS

10 In another aspect, the present invention provides pharmaceutical compositions comprising at least one of the previously described ADNF polypeptides comprising at least one D-amino acid within the active core site in an amount sufficient to exhibit neuroprotective/neurotrophic activity, and a pharmaceutically acceptable diluent, carrier or excipient. The pharmaceutical compositions comprising one of the previously
15 described ADNF polypeptides are particularly useful as oral agents, as they have stability in the gastrointestinal tract and can be absorbed without change. Moreover, by using mixtures of ADNF polypeptides in L-form and in D-form for producing pharmaceutical compositions, pharmaceutical compositions possessing varying dose response properties can be obtained. These pharmaceutical compositions are useful, *inter alia*, in targeting
20 different receptors that may have different affinities for ADNF polypeptides, or to provide custom tailored drug treatment regime for individuals affected by, *e.g.*, neurodegenerative disorders.

In one embodiment, the pharmaceutical composition comprises a pharmaceutically acceptable excipient and an ADNF polypeptide, wherein the ADNF
25 polypeptide is a member selected from the group consisting of: (a) an ADNF I polypeptide comprising an active core site having the following amino acid: Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1); (b) an ADNF III polypeptide comprising an active core site having the following amino acid: Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2); and (c) a mixture of the ADNF I polypeptide or part (a) and the ADNF III
30 polypeptide of part (b); wherein at least one of the ADNF I polypeptide and the ADNF III polypeptide comprises an active core site comprising at least one D-amino acid, preferably at the N-terminus and/or the C-terminus of the active core site.

In another embodiment, the pharmaceutical composition comprises a pharmaceutically acceptable excipient and an ADNF I polypeptide, wherein the active core site of the ADNF I polypeptide comprises at least one D-amino acid, preferably at the N-terminus and/or the C-terminus of the active core site. The previous discussion
5 pertaining to the location and the number of D-amino acids within the active core site of ADNF I, as well as the discussion of additional D- and/or L-amino acids added on to the active site of the ADNF I polypeptide is fully applicable, and thus, will not be repeated with respect to this particular embodiment of the invention.

In yet another embodiment, the pharmaceutical composition comprises a
10 pharmaceutically acceptable excipient and an ADNF III polypeptide, wherein the active core site of the ADNF III polypeptide comprises at least one D-amino acid, preferably at the N-terminus and/or the C-terminus of the active core site. The previous discussion pertaining to the location and the number of D-amino acids within the active core site of ADNF III, as well as the discussion of additional D- and/or L-amino acids added on to the
15 active site of the ADNF III polypeptide is fully applicable, and thus, will not be repeated with respect to this particular embodiment of the invention.

In yet another embodiment, the pharmaceutical composition comprises a pharmaceutically acceptable excipient and a mixture of an ADNF I polypeptide and an ADNF III polypeptide, wherein at least one of the ADNF I and the ADNF III
20 polypeptides comprises an active core site comprising at least one D-amino acid. The previous discussion pertaining to the location and the number of D-amino acids within the active core site of ADNF I or ADNF III, as well as the discussion of additional D- and/or L-amino acids added on to the active site of the ADNF I or ADNF III polypeptide is fully applicable, and thus, will not be repeated with respect to this particular embodiment of the
25 invention. In yet another embodiment, the pharmaceutical composition comprises an ADNF I polypeptide comprising all D-amino acids and an ADNF III polypeptide comprising all L-amino acids. In yet another embodiment, the pharmaceutical composition comprises an ADNF I polypeptide comprising all L-amino acids and an ADNF III polypeptide comprising all D-amino acids. In yet another embodiment, the
30 pharmaceutical composition comprises an ADNF I polypeptide comprising all D-amino acids and an ADNF III polypeptide comprising all D-amino acids.

In a pharmaceutical composition, any one or more of the ADNF I polypeptide described herein can be mixed with any one or more of the ADNF III polypeptide described herein. A mixture of an ADNF I polypeptide and an ADNF III

polypeptide can be a blend of two or more of these polypeptides. A mixture of an ADNF I polypeptide and an ADNF III polypeptide can also refer to one or more of ADNF I polypeptides that are coupled to one or more of ADNF III polypeptides. For example, an ADNF I polypeptide can be covalently linked to an ADNF III polypeptide. A mixture of
5 an ADNF I polypeptide and an ADNF III polypeptide can be prepared as a single composition and can be administered to a subject. Alternatively, an ADNF I polypeptide and an ADNF III polypeptide can be prepared as separate compositions and can be administered simultaneously or sequentially to a subject. Furthermore, different proportions of an ADNF I polypeptide and an ADNF III polypeptide can be administered
10 to a subject. For example, in a mixture the ratio of an ADNF I polypeptide and an ADNF III polypeptide can be in the range of 1:100 to 100:1, 1:10 to 10:1, or 1:2 to 2:1.

The pharmaceutical compositions of the present invention are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences* (17th ed. 1985)), which is
15 incorporated herein by reference. A brief review of methods for drug delivery is described in, e.g., Langer, *Science* 249:1527-1533 (1990), which is incorporated herein by reference. In addition, the pharmaceutical compositions comprising peptides and proteins are described in, e.g., *Therapeutic Peptides and Proteins Formulations, Processing, and Delivery Systems*, by Ajay K. Banga, Technomic Publishing Company,
20 Inc., Lancaster, PA (1995).

In a preferred embodiment, the pharmaceutical composition of the present invention is formulated for oral administration. In this embodiment, it is preferred that ADNF polypeptides comprising all D-amino acids are used. A pharmaceutically acceptable nontoxic composition is formed by incorporating any of normally employed
25 excipients, and generally 10-95% of active ingredient and more preferably at a concentration of 25%-75%. Furthermore, to improve oral absorption of ADNF polypeptides, various carrier systems, such as nanoparticles, microparticles, liposomes, phospholipids, emulsions, erythrocytes, etc. can be used. The oral agents comprising ADNF polypeptides of the invention can be in any suitable form for oral administration,
30 such as liquid, tablets, capsules, or the like. The oral formulations can be further coated or treated to prevent or reduce dissolution in stomach. See, e.g., *Therapeutic Peptides and Proteins, Formulation, Processing, and Delivery Systems*, by A.K. Banga, Technomic Publishing Company, Inc., 1995.

Furthermore, the ADNF polypeptides comprising at least one D-amino acid within the active core sites are embodied in pharmaceutical compositions intended for parenteral, topical, oral, sublingual, gavage, or local administration. For example, the pharmaceutical compositions are administered parenterally, *e.g.*, intravenously, subcutaneously, intradermally, or intramuscularly, or intranasally. Thus, the invention provides compositions for parenteral administration that comprise a solution of a mixture of ADNF polypeptides, dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used including, for example, water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques or, they may be sterile filtered. The resulting aqueous solutions may be packaged for use as is or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions including pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, such as, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.*

For aerosol administration, ADNF polypeptides comprising at least one D-amino acid within the active core sites are preferably supplied in finely divided form along with a surfactant and propellant. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. A carrier can also be included, as desired, as with, *e.g.*, lecithin for intranasal delivery.

For solid compositions, conventional nontoxic solid carriers may be used. Solid carriers include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

Small polypeptides including SALLRSIPA and NAPVSIPQ cross the blood brain barrier. For longer polypeptides that do not cross blood the brain barrier, methods of administering proteins to the brain are well known. For example, proteins, polypeptides, other compounds and cells can be delivered to the mammalian brain via

intracerebroventricular (ICV) injection or via a cannula (*see, e.g., Motta & Martini, Proc. Soc. Exp. Biol. Med.* 168:62-64 (1981); Peterson *et al., Biochem. Pharmacol.* 31:2807-2810 (1982); Rzepczynski *et al., Metab. Brain Dis.* 3:211-216 (1988); Leibowitz *et al., Brain Res. Bull.* 21:905-912 (1988); Sramka *et al., Stereotact. Funct. Neurosurg.* 58:79-83 (1992); Peng *et al., Brain Res.* 632:57-67 (1993); Chem *et al., Exp. Neurol.* 125:72-81 (1994); Nikkiah *et al., Neuroscience* 63:57-72 (1994); Anderson *et al., J. Comp. Neurol.* 357:296-317 (1995); and Brecknell & Fawcett, *Exp. Neurol.* 138:338-344 (1996)). In particular, cannulas can be used to administer neurotrophic factors to mammals (*see, e.g., Motta & Martini, Proc. Soc. Exp. Biol. Med.* 168:62-64 (1981) (neurotensin); Peng *et al., Brain Res.* 632:57-67 (1993) (NGF); Anderson *et al., J. Comp. Neurol.* 357:296-317 (1995) (BDNF, NGF, neurotrophin-3).

Alternatively, longer ADFN polypeptides that do not cross blood brain barrier can be coupled with a material which assists the ADFN polypeptide to cross the blood brain barrier and to traverse the plasma membrane of a cell, or the membrane of an intra-cellular compartment such as the nucleus. Cellular membranes are composed of lipid-protein bilayers that are freely permeable to small, nonionic lipophilic compounds and are inherently impermeable to polar compounds, macromolecules, and therapeutic or diagnostic agents. However, proteins and other compounds such as liposomes have been described, which have the ability to translocate polypeptides such as ADFN polypeptides across a cell membrane.

For example, "membrane translocation polypeptides" have amphiphilic or hydrophobic amino acid subsequences that have the ability to act as membrane-translocating carriers. In one embodiment, homeodomain proteins have the ability to translocate across cell membranes. The shortest internalizable peptide of a homeodomain protein, Antennapedia, was found to be the third helix of the protein, from amino acid position 43 to 58 (*see, e.g., Prochiantz, Current Opinion in Neurobiology* 6:629-634 (1996)). Another subsequence, the hydrophobic domain of signal peptides, was found to have similar cell membrane translocation characteristics (*see, e.g., Lin et al., J. Biol. Chem.* 270:14255-14258 (1995)).

Examples of peptide sequences which can be linked to a ADFN polypeptide of the invention, for facilitating uptake of ADFN polypeptides into cells, include, but are not limited to: an 11 amino acid peptide of the tat protein of HIV (*see Schwarze et al., Science* 285:1569-1572 (1999)); a 20 residue peptide sequence which corresponds to amino acids 84-103 of the p16 protein (*see Fahraeus et al., Current*

Biology 6:84 (1996)); the third helix of the 60-amino acid long homeodomain of Antennapedia (Derossi *et al.*, *J. Biol. Chem.* 269:10444 (1994)); the h region of a signal peptide such as the Kaposi fibroblast growth factor (K-FGF) h region (Lin *et al.*, *supra*); or the VP22 translocation domain from HSV (Elliot & O'Hare, *Cell* 88:223-233 (1997)).

- 5 Other suitable chemical moieties that provide enhanced cellular uptake may also be chemically linked to ADNF polypeptides.

Toxin molecules also have the ability to transport polypeptides across cell membranes. Often, such molecules are composed of at least two parts (called "binary toxins"): a translocation or binding domain or polypeptide and a separate toxin domain or polypeptide. Typically, the translocation domain or polypeptide binds to a cellular receptor, and then the toxin is transported into the cell. Several bacterial toxins, including *Clostridium perfringens* iota toxin, diphtheria toxin (DT), *Pseudomonas* exotoxin A (PE), pertussis toxin (PT), *Bacillus anthracis* toxin, and pertussis adenylate cyclase (CYA), have been used in attempts to deliver peptides to the cell cytosol as internal or amino-terminal fusions (Arora *et al.*, *J. Biol. Chem.*, 268:3334-3341 (1993); Perelle *et al.*, *Infect. Immun.*, 61:5147-5156 (1993); Stenmark *et al.*, *J. Cell Biol.* 113:1025-1032 (1991); Donnelly *et al.*, *PNAS* 90:3530-3534 (1993); Carbonetti *et al.*, *Abstr. Annu. Meet. Am. Soc. Microbiol.* 95:295 (1995); Sebo *et al.*, *Infect. Immun.* 63:3851-3857 (1995); Klimpel *et al.*, *PNAS U.S.A.* 89:10277-10281 (1992); and Novak *et al.*, *J. Biol. Chem.* 267:17186-17193 (1992)).

Such subsequences can be used to translocate ADNF polypeptides across a cell membrane. ADNF polypeptides can be conveniently fused to or derivatized with such sequences. Typically, the translocation sequence is provided as part of a fusion protein. Optionally, a linker can be used to link the ADNF polypeptides and the translocation sequence. Any suitable linker can be used, *e.g.*, a peptide linker.

The ADNF polypeptides can also be introduced into an animal cell, preferably a mammalian cell, via a liposomes and liposome derivatives such as immunoliposomes. The term "liposome" refers to vesicles comprised of one or more concentrically ordered lipid bilayers, which encapsulate an aqueous phase. The aqueous phase typically contains the compound to be delivered to the cell, *i.e.*, an ADNF polypeptide.

The liposome fuses with the plasma membrane, thereby releasing the ADNF polypeptides into the cytosol. Alternatively, the liposome is phagocytosed or taken up by the cell in a transport vesicle. Once in the endosome or phagosome, the

liposome either degrades or fuses with the membrane of the transport vesicle and releases its contents.

In current methods of drug delivery via liposomes, the liposome ultimately becomes permeable and releases the encapsulated compound (in this case, an ADNF polypeptide) at the target tissue or cell. For systemic or tissue specific delivery, this can be accomplished, for example, in a passive manner wherein the liposome bilayer degrades over time through the action of various agents in the body. Alternatively, active drug release involves using an agent to induce a permeability change in the liposome vesicle. Liposome membranes can be constructed so that they become destabilized when the environment becomes acidic near the liposome membrane (*see, e.g., PNAS* 84:7851 (1987); *Biochemistry* 28:908 (1989)). When liposomes are endocytosed by a target cell, for example, they become destabilized and release their contents. This destabilization is termed fusogenesis. Dioleoylphosphatidylethanolamine (DOPE) is the basis of many "fusogenic" systems.

Such liposomes typically comprise an ADNF polypeptide and a lipid component, *e.g.*, a neutral and/or cationic lipid, optionally including a receptor-recognition molecule such as an antibody that binds to a predetermined cell surface receptor or ligand (*e.g.*, an antigen). A variety of methods are available for preparing liposomes as described in, *e.g.*, Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, 4,946,787, PCT Publication No. WO 91/17424, Deamer & Bangham, *Biochim. Biophys. Acta* 443:629-634 (1976); Fraley, *et al.*, *PNAS* 76:3348-3352 (1979); Hope *et al.*, *Biochim. Biophys. Acta* 812:55-65 (1985); Mayer *et al.*, *Biochim. Biophys. Acta* 858:161-168 (1986); Williams *et al.*, *PNAS* 85:242-246 (1988); *Liposomes* (Ostro (ed.), 1983, Chapter 1); Hope *et al.*, *Chem. Phys. Lip.* 40:89 (1986); Gregoriadis, *Liposome Technology* (1984) and Lasic, *Liposomes: from Physics to Applications* (1993)). Suitable methods include, for example, sonication, extrusion, high pressure/homogenization, microfluidization, detergent dialysis, calcium-induced fusion of small liposome vesicles and ether-fusion methods, all of which are well known in the art.

In certain embodiments of the present invention, it is desirable to target the liposomes of the invention using targeting moieties that are specific to a particular cell type, tissue, and the like. Targeting of liposomes using a variety of targeting moieties (*e.g.*, ligands, receptors, and monoclonal antibodies) has been previously described (*see,*

e.g., U.S. Patent Nos. 4,957,773 and 4,603,044). Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes lipid components, e.g., phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid derivatized bleomycin. Antibody targeted liposomes can be constructed using, for instance, liposomes which incorporate protein A (see Renneisen *et al.*, *J. Biol. Chem.*, 265:16337-16342 (1990) and Leonetti *et al.*, *PNAS* 87:2448-2451 (1990)).

Alternatively, nucleic acids encoding ADNF can also be used to provide a therapeutic dose of ADNF polypeptides. These nucleic acids can be inserted into any of a number of well-known vectors for the transfection of target cells and organisms. For example, nucleic acids are delivered as DNA plasmids, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures, see Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1988); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds) (1995); and Yu *et al.*, *Gene Therapy* 1:13-26 (1994).

Methods of non-viral delivery of nucleic acids include lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in, e.g., U.S. Patent No. 5,049,386, U.S. Patent No. 4,946,787; and U.S. Patent No. 4,897,355) and lipofection reagents are sold commercially (e.g., Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424, WO 91/16024. Delivery can be to cells (*ex vivo* administration) or target tissues (*in vivo* administration).

In therapeutic applications, ADNF polypeptides of the invention are administered to a patient in an amount sufficient to reduce neuronal cell death associated with various disorders, to reduce oxidative stress in a patient, or to reduce a condition

associated with fetal alcohol syndrome in a subject *in utero*. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend on, for example, the particular ADNF polypeptide employed, the conditions to be treated, the type of neuronal cell death or damage to be prevented, the manner of administration, the weight and general state of health of the patient, and the judgment of the prescribing physician. For example, for the prevention or reduction of neuronal cell death, an amount of ADNF polypeptides falling within the range of a 1µg to 50 µg, preferably 1µg to 10µg dose given orally once a day per mouse (*e.g.*, in the evening) would be a therapeutically effective amount. This dose is based on the average body weight of mice, and an appropriate dose for human can be extrapolated based on the average weight of human.

IV. METHODS FOR REDUCING NEURONAL CELL DEATH

In another aspect, the present invention provides a method for reducing neuronal cell death, the method comprising contacting neuronal cells with an ADNF polypeptide in an amount sufficient to reduce neuronal cell death, wherein the ADNF polypeptide comprises at least one D-amino acid within its active core site, preferably at the N-terminus and/or the C-terminus of the active core site. In this method, the ADNF polypeptide can be an ADNF I polypeptide, an ADNF III polypeptide, or mixtures thereof.

In one embodiment, the method comprises contacting neuronal cells with an ADNF polypeptide, wherein the ADNF polypeptide is a member selected from the group consisting of: (a) an ADNF I polypeptide comprising an active core site having the following amino acid: Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1); (b) an ADNF III polypeptide comprising an active core site having the following amino acid: Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2); and (c) a mixture of the ADNF I polypeptide or part (a) and the ADNF III polypeptide of part (b); wherein at least one of the ADNF I polypeptide and the ADNF III polypeptide comprises an active core site comprising at least one D-amino acid.

In another embodiment, the method comprises contacting neuronal cells with an ADNF I polypeptide, wherein the active core site of the ADNF I polypeptide comprises at least one D-amino acid, preferably at the N-terminus and/or the C-terminus of the active core site. The previous discussion pertaining to the location and the number

of D-amino acids within the active core site of ADNF I, as well as the discussion of additional D- and/or L-amino acids added on to the active site of the ADNF I polypeptide is fully applicable, and thus, will not be repeated with respect to this particular embodiment of the invention.

5 In yet another embodiment, the method comprises contacting neuronal cells with an ADNF III polypeptide, wherein the active core site of the ADNF III polypeptide comprises at least one D-amino acid, preferably at the N-terminus and/or the C-terminus of the active core site. The previous discussion pertaining to the location and the number of D-amino acids within the active core site of ADNF III, as well as the
10 discussion of additional D- and/or L-amino acids added on to the active site of the ADNF III polypeptide is fully applicable, and thus, will not be repeated with respect to this particular embodiment of the invention.

In yet another embodiment, the method comprises contacting neuronal cells with a mixture of an ADNF I polypeptide and an ADNF III polypeptide, wherein at
15 least one of the ADNF I polypeptide and the ADNF III polypeptide comprises an active core site comprising at least one D-amino acid. The previous discussion pertaining to the location and the number of D-amino acids within the active core site of ADNF I or ADNF III, as well as the discussion of additional D- and/or L-amino acids added on to the active site of the ADNF I polypeptide or ADNF III polypeptide is fully applicable, and thus, will
20 not be repeated with respect to this particular embodiment of the invention.

ADNF polypeptides of the present invention can be used in the treatment of neurological disorders and for the prevention of neuronal cell death. For example, ADNF polypeptides of the present invention can be used to prevent the death of neuronal cells including, but not limited to, spinal cord neurons, hippocampal neurons, cerebral
25 cortical neurons and cholinergic neurons. More particularly, ADNF polypeptides of the present invention can be used in the prevention of cell death associated with (1) gp120, the envelope protein from HIV; (2) N-methyl-D-aspartic acid (excito-toxicity); (3) tetrodotoxin (blockage of electrical activity); and (4) β -amyloid peptide, a substance related to neuronal degeneration in Alzheimer's disease.

30 As such, the ADNF polypeptides of the present invention can be used to reduce gp120-induced neuronal cell death by administering an effective amount of an ADNF polypeptide of the present invention to a patient infected with the HIV virus. The ADNF polypeptides of the present invention can also be used to reduce neuronal cell death associated with excito-toxicity induced by N-methyl-D-aspartate stimulation, the

method comprising contacting neuronal cells with an ADFN polypeptide of the present invention in an amount sufficient to prevent neuronal cell death. The ADFN polypeptides of the present invention can also be used to reduce cell death induced by the β -amyloid peptide in a patient afflicted or impaired with Alzheimer's disease, the method

- 5 comprising administering to the patient an ADFN polypeptide of the present invention in an amount sufficient to prevent neuronal cell death. The ADFN polypeptides can also be used to alleviate learning impairment produced by cholinergic blockage in a patient afflicted or impaired with Alzheimer's disease. For example, ADFN polypeptides can be used to improve short-term and/or reference memory in Alzheimer's patients.

- 10 Similarly, it will be readily apparent to those of skill in the art that the ADFN polypeptides of the present invention can be used in a similar manner to prevent neuronal cell death associated with a number of other neurological diseases and deficiencies. Pathologies that would benefit from therapeutic and diagnostic applications of this invention include conditions (diseases and insults) leading to neuronal cell death and/or sub-lethal neuronal pathology including, for example, the following:

- 15 diseases of central motor systems including degenerative conditions affecting the basal ganglia (Huntington's disease, Wilson's disease, striatonigral degeneration, corticobasal ganglionic degeneration), Tourette's syndrome, Parkinson's disease, progressive supranuclear palsy, progressive bulbar palsy, familial spastic paraplegia, spinomuscular atrophy, ALS and variants thereof, dentatorubral atrophy, olivo-pontocerebellar atrophy, paraneoplastic cerebellar degeneration, and dopamine toxicity;
- 20 diseases affecting sensory neurons such as Friedreich's ataxia, diabetes, peripheral neuropathy, retinal neuronal degeneration;
- 25 diseases of limbic and cortical systems such as cerebral amyloidosis, Pick's atrophy, Retts syndrome;
- neurodegenerative pathologies involving multiple neuronal systems and/or brainstem including Alzheimer's disease, AIDS-related dementia, Leigh's disease, diffuse Lewy body disease, epilepsy, multiple system atrophy, Guillain-Barre syndrome,
- 30 lysosomal storage disorders such as lipofuscinosis, late-degenerative stages of Down's syndrome, Alper's disease, vertigo as result of CNS degeneration;
- pathologies associated with developmental retardation and learning impairments, and Down's syndrome, and oxidative stress induced neuronal death;

pathologies arising with aging and chronic alcohol or drug abuse including, for example, with alcoholism the degeneration of neurons in locus coeruleus, cerebellum, cholinergic basal forebrain; with aging degeneration of cerebellar neurons and cortical neurons leading to cognitive and motor impairments; and with chronic
5 amphetamine abuse degeneration of basal ganglia neurons leading to motor impairments; pathological changes resulting from focal trauma such as stroke, focal ischemia, vascular insufficiency, hypoxic-ischemic encephalopathy, hyperglycemia, hypoglycemia, closed head trauma, or direct trauma;

pathologies arising as a negative side-effect of therapeutic drugs and
10 treatments (e.g., degeneration of cingulate and entorhinal cortex neurons in response to anticonvulsant doses of antagonists of the NMDA class of glutamate receptor).

Other ADNF polypeptides (including their alleles, polymorphic variants, species homologs and subsequences thereof) that reduce neuronal cell death can be screened using the various methods described in U.S.S.N. 60/037,404, filed February 7,
15 1997 (published as WO98/35042), and U.S.S.N. 09/187,330 filed November 6, 1998, both of which are incorporated herein by reference. For example, it will be readily apparent to those skilled in the art that using the teachings set forth above with respect to the design and synthesis of ADNF polypeptides and the assays described herein, one of ordinary skill in the art can identify other biologically active ADNF polypeptides
20 comprising at least one D-amino acid within their active core sites. For example, Brenneman *et al.*, *Nature* 335:639-642 (1988), and Dibbern *et al.*, *J. Clin. Invest.* 99:2837-2841 (1997), incorporated herein by reference, teach assays that can be used to screen ADNF polypeptides that are capable of reducing neuronal cell death associated with envelope protein (gp120) from HIV. Also, Brenneman *et al.*, *Dev. Brain Res.* 51:63-
25 68 (1990), and Brenneman & Gozes, *J. Clin. Invest.* 97:2299-2307 (1996), incorporated herein by reference, teach assays that can be used to screen ADNF polypeptides which are capable of reducing neuronal cell death associated with excitotoxicity induced by stimulation by *N*-methyl-D-aspartate. Other assays described in, e.g., WO98/35042 can also be used to identify other biologically active ADNF polypeptides comprising at least
30 one D-amino acid within their active core sites.

Moreover, ADNF polypeptides that reduce neuronal cell death can be screened *in vivo*. For example, the ability of ADNF polypeptides that can protect against learning and memory deficiencies associated with cholinergic blockade can be tested. For example, cholinergic blockade can be obtained in rats by administration of the

cholinotoxin AF64A, and ADFN polypeptides can be administered intranasally and the water maze experiments can be performed (Gozes *et al.*, *Proc. Natl. Acad. Sci. USA* 93:427-432 (1996), the teachings of which are incorporated herein by reference). Animals treated with efficacious ADFN polypeptides would show improvement in their learning and memory capacities compared to the control.

Furthermore, the ability of ADFN polypeptides that can protect or reduce neuronal cell death associated with Alzheimer's disease can be screened *in vivo*. For these experiments, apolipoprotein E (ApoE)-deficient homozygous mice can be used (Plump *et al.*, *Cell* 71:343-353 (1992); Gordon *et al.*, *Neuroscience Letters* 199:1-4 (1995); Gozes *et al.*, *J. Neurobiol.* 33:329-342 (1997)), the teachings of which are incorporated herein by reference.

V. METHODS FOR REDUCING OXIDATIVE STRESS

In yet another aspect, the present invention provides methods for treating oxidative stress in a patient by administering to the patient an ADFN polypeptide in an amount sufficient to prevent or reduce oxidative stress, wherein the ADFN polypeptide comprises at least one D-amino acid within its active core site, preferably at the N-terminus and/or the C-terminus of the active core site. Oxidative stress has been implicated in several neurodegenerative diseases in humans (Cassarmino & Bennett, *Brain Res. Reviews* 29:1-25 (1999)). Moreover, oxidative stress produced from alcohol administration has been associated with fetal death and abnormalities (*e.g.*, conditions associated with fetal alcohol syndrome). *See, e.g.*, Henderson *et al.*, *Alcoholism: Clinical and Experimental Research* 19:714-720 (1995). In these methods, the ADFN polypeptide can be an ADFN I polypeptide, an ADFN III polypeptide, or mixtures thereof. By using the ADFN polypeptides of the present invention, oxidative stress associated with various clinical conditions can be reduced.

In one embodiment, the method comprises treating oxidative stress in a patient with an ADFN polypeptide, wherein the ADFN polypeptide is a member selected from the group consisting of: (a) an ADFN I polypeptide comprising an active core site having the following amino acid: Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1); (b) an ADFN III polypeptide comprising an active core site having the following amino acid: Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2); and (c) a mixture of the ADFN I polypeptide or part (a) and the ADFN III polypeptide of part (b); wherein at least one of

the ADNF I polypeptide and the ADNF III polypeptide comprises an active core site comprising at least one D-amino acid, preferably at the N-terminus and/or the C-terminus of the active core site.

In another embodiment, the method comprises treating oxidative stress in a patient with an ADNF I polypeptide, wherein the active core site of the ADNF I polypeptide comprises at least one D-amino acid, preferably at the N-terminus and/or the C-terminus of the active core site. The previous discussion pertaining to the location and the number of D-amino acids within the active core site of ADNF I, as well as the discussion of additional D- and/or L-amino acids added on to the active site of the ADNF I polypeptide is fully applicable, and thus, will not be repeated with respect to this particular embodiment of the invention.

In yet another embodiment, the method comprises treating oxidative stress in a patient with an ADNF III polypeptide, wherein the active core site of the ADNF III polypeptide comprises at least one D-amino acid, preferably at the N-terminus and/or the C-terminus of the active core site. The previous discussion pertaining to the location and the number of D-amino acids within the active core site of ADNF III, as well as the discussion of additional D- and/or L-amino acids added on to the active site of the ADNF III polypeptide is fully applicable, and thus, will not be repeated with respect to this particular embodiment of the invention.

In yet another embodiment, the method comprises treating oxidative stress in a patient with a mixture of an ADNF I polypeptide and an ADNF III polypeptide, wherein at least one of the ADNF I and the ADNF III polypeptide comprises an active core site comprising at least one D-amino acid. The previous discussion pertaining to the location and the number of D-amino acids within the active core site of ADNF I or ADNF III, as well as the discussion of additional D- and/or L-amino acids added on to the active site of the ADNF I or ADNF III polypeptide is fully applicable, and thus, will not be repeated with respect to this particular embodiment of the invention.

Other ADNF polypeptides (including their alleles, polymorphic variants, species homologs and subsequences thereof) that are effective in reducing oxidative stress can be screened using primary neurons. For example, cultured embryonic neurons (E18) rat hippocampal neurons can be treated with, *e.g.*, 0.5 μM FeSO_4 to induce oxidative stress. The degree of oxidative stress can be quantified by cell counting and/or morphological criteria. Furthermore, apoptosis induced by oxidative stress results in

nuclear condensation and DNA fragmentation. Apoptotic nuclei can be measured by counting cells in culture stained with the fluorescent DNA-binding dye, *e.g.*, Hoescht 33342. *See Glazner et al., Society for Neuroscience 27th Annual Meeting*, Abstracts vol. 23, part 2 (1997). To screen ADFN polypeptides comprising at least one D-amino acid that can reduce oxidative stress *in vitro*, FeSO₄ treated neurons can be contacted with various ADFN polypeptides comprising D-amino acids for sufficient time (*e.g.*, 24 hours). Cells with apoptotic nuclei can be quantified as described above. ADFN polypeptides comprising at least one D-amino acid that reduce the quantity of apoptotic nucleic compared to control (*e.g.*, cells untreated with ADFN polypeptides) can be used to treat oxidative stress in a patient.

Other ADFN polypeptides that are effective in reducing oxidative stress can also be screened using *in vivo* assays. For example, ethanol consumption is known to cause oxidative stress *in vivo*. In the human body, ethanol is metabolized into cytotoxic acetaldehyde by alcohol dehydrogenase enzyme in the liver and acetaldehyde is oxidized to acetate by aldehyde oxidase or xanthine oxidase giving rise to free radicals or reactive oxygen species (ROS). *See, e.g., Schlorff et al., Alcohol 17:95-105 (1999)*. Thus, ethanol consumption can be used to induce oxidative stress in *in vivo* animal models (*e.g.*, rat, mouse, human, *etc.*). Thereafter, animals suffering from ethanol induced oxidative stress can be used as models to screen other ADFN polypeptides comprising at least one D-amino acid that can reduce the level of oxidative stress.

The level of oxidative stress of cells and tissues of *in vivo* animal models can be measured using a number of assays known in the art. For example, protocols described in Schlorff *et al.* (1999), *supra*, can be used to measure effects of rat ethanol ingestion on lipid peroxidation in plasma (*e.g.*, plasma malondialdehyde) and changes in antioxidant system (*e.g.*, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, *etc.*). Effective ADFN polypeptides are those that prevent or reduce changes in lipid peroxidation in plasma or on antioxidant system in ethanol ingested animal models compared to control (*e.g.*, animal models untreated with ADFN polypeptides). In another example, fetal death and abnormalities (*e.g.*, conditions associated with fetal alcohol syndrome) are considered a severe form of oxidative stress produced from alcohol administration (Henderson *et al., Alcoholism: Clinical and Experimental Research 19:714-720 (1995)*). Therefore, a well established model (*e.g.*, mice) for fetal alcohol syndrome can also be used to screen for other ADFN polypeptides that can reduce oxidative stress. The use of this model for fetal alcohol syndrome and

methods for reducing a condition associated with fetal alcohol syndrome are described in detail below.

VI. METHODS FOR REDUCING A CONDITION ASSOCIATED WITH FETAL ALCOHOL SYNDROME

In yet another aspect, the present invention provides a method for reducing a condition associated with fetal alcohol syndrome in a subject who is exposed to alcohol *in utero*, the method comprising administering to the subject an ADNF polypeptide in an amount sufficient to reduce the condition associated with fetal alcohol syndrome, wherein the ADNF polypeptide comprises an active core site comprising at least one D-amino acid, preferably at the N-terminus and/or the C-terminus of the active core site. In this method, the ADNF polypeptide can be an ADNF I polypeptide, an ADNF III polypeptide, or mixtures thereof.

Treatment of a well-characterized model for FAS (e.g., C57B1/6J mouse strain) with an ADNF polypeptide comprising at least one D-amino acid within an active core site reduces or prevents alcohol induced fetus death, body and brain weight reduction, and VIP mRNA reduction. Similarly, the human embryo, fetus, or subject can be protected from alcohol induced effects by administering an ADNF polypeptide directly to the embryo, fetus, or subject, or by administering the ADNF polypeptide indirectly to the fetus by administering it to the mother. Preferably, ADNF polypeptides are orally administered.

In one embodiment, the method comprises administering to a subject who is exposed to alcohol *in utero* with an ADNF polypeptide, wherein the ADNF polypeptide is a member selected from the group consisting of: (a) an ADNF I polypeptide comprising an active core site having the following amino acid: Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1); (b) an ADNF III polypeptide comprising an active core site having the following amino acid: Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2); and (c) a mixture of the ADNF I polypeptide or part (a) and the ADNF III polypeptide of part (b); wherein at least one of the ADNF I polypeptide and the ADNF III polypeptide comprises an active core site comprising at least one D-amino acid.

In another embodiment, the method comprises administering to a subject who is exposed to alcohol *in utero* with an ADNF I polypeptide, wherein the active core site of the ADNF I polypeptide comprises at least one D-amino acid, preferably at the N-terminus and/or the C-terminus of the active core site. The previous discussion pertaining

to the location and the number of D-amino acids within the active core site of ADNF I, as well as the discussion of additional D- and/or L-amino acids added on to the active site of the ADNF I polypeptide is fully applicable, and thus, will not be repeated with respect to this particular embodiment of the invention.

5 In yet another embodiment, the method comprises administering to a subject who is exposed to alcohol *in utero* with an ADNF III polypeptide, wherein the active core site of the ADNF III polypeptide comprises at least one D-amino acid, preferably at the N-terminus and/or the C-terminus of the active core site. The previous discussion pertaining to the location and the number of D-amino acids within the active
10 core site of ADNF III, as well as the discussion of additional D- and/or L-amino acids added on to the active site of the ADNF III polypeptide is fully applicable, and thus, will not be repeated with respect to this particular embodiment of the invention.

In yet another embodiment, the method comprises administering to a subject who is exposed to alcohol *in utero* with a mixture of an ADNF I polypeptide and
15 an ADNF III polypeptide, wherein at least one of the ADNF I and the ADNF III polypeptide comprises an active core site comprising at least one D-amino acid. The previous discussion pertaining to the location and the number of D-amino acids within the active core site of ADNF I or ADNF III, as well as the discussion of additional D- and/or L-amino acids added on to the active site of the ADNF I or the ADNF III polypeptide is
20 fully applicable, and thus, will not be repeated with respect to this particular embodiment of the invention.

Other ADNF polypeptides (including their alleles, polymorphic variants, species homologs and subsequences thereof) comprising at least one D-amino acid within their active core sites that reduce a condition associated with fetal alcohol syndrome can
25 be screened using a well-characterized animal model for FAS. For example, the C57B1/6J mouse strain can be used. Previous work with this strain has defined the effects of dosage and embryonic timing on maternal serum alcohol levels and embryonic effects (Webster *et al.*, *Neurobehav. Tox.*, 2:227-34 (1980), incorporated herein by reference). Intra-peritoneal treatment allows for defined and reproducible dosages.

30 Acute (single) dosages of alcohol can reproduce the phenotype of FAS (Webster *et al.*, (1980), *supra*). Since treatment on E8 results in the highest rate of fetal anomalies and demises, and vasoactive intestinal peptide's growth regulating effects on the embryo are limited to the early post-implantation period of embryogenesis, E8 can be chosen as a test for screening neuroprotective ADNF polypeptides comprising at least one D-amino acid

within their active core sites. The mice can be injected with 25% ethyl alcohol in saline (v/v) or vehicle alone at, *e.g.*, 0.030 ml/g maternal body weight at, *e.g.*, 9:00a.m. on E8 (embryonic gestation day 8). Effective ADNF polypeptides can be screened by pretreating the mice 30 minutes prior to alcohol administration. In one embodiment, the
5 dose for nasal administration for an ADNF polypeptide is about 1 μ g-50 μ g, preferably about 1 μ g-10 μ g/mouse. This dose is based on the average body weight of mice, and an appropriate dose for human can be extrapolated based on the average body weight of human.

Various parameters can be measured to determine if an ADNF polypeptide
10 comprising at least one D-amino acid within its active core site reduces a condition associated with fetal alcohol syndrome. For example, a number of fetal demises (*i.e.*, death) can be compared between the control (*e.g.*, untreated with ADNF polypeptides) and a group treated with ADNF polypeptides comprising at least one D-amino acid within their active core sites. In another example, the fetal weight and fetal brain weight in the
15 surviving E18 fetuses can be compared. In another example, the level of VIP mRNA can be compared between the control and a group treated with ADNF polypeptides comprising at least one D-amino acid within their active core sites. In another example, the degree of learning deficits can be compared between the control and a group treated with ADNF polypeptides. In another example, the glutathione level in the control and the
20 treated group can be compared.

EXAMPLES

A. *In vitro* experiments

Dissociated cerebral cortical cultures prepared as described (Brenneman &
25 Gozes, *J. Clin. Invest.* 97:2299-2307 (1996)) were used to compare the survival-promoting actions of ADNF I and ADNF III derived peptides. Comparisons were made with the D-form of the peptide and in combination with the L-form of the peptides. The test paradigm consisted of the addition of the test peptide in cultures that were co-treated with tetrodotoxin (TTX). TTX produced an apoptotic death in these cultures and is used
30 as a model substance to demonstrate efficacy against this "programmed cell death" and all other means that produce this type of death mechanism. The duration of the test period was 5 days, and neurons were counted and identified by characteristic morphology

and by confirmation with an immunocytochemical marker for neurons: neuron specific enolase.

As shown in Figure 1, the D- and L-forms of SALLRSIPA (SAL) were identical in both potency and efficacy in preventing neuronal cell death associated with electrical blockade with TTX. Each point is the mean of at least three determinations, the error bars are the standard errors. Similarly, the D- and L-forms of NAPVSIPQ (NAP) were very similar, with each exhibiting a complex dose response with two apparent maxima (Figure 2A). Unless indicated as otherwise, L-SAL and D-SAL refer to a peptide having an amino acid sequence of Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1) comprising all L-amino acids or all D-amino acids, respectively. Also, L-NAP and D-NAP refer to a peptide having an amino acid sequence of Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2) comprising all L-amino acids or all D-amino acids, respectively.

In Figure 2B, the effect of an ADNF peptide that has amino acid residues in both L-form and in D-form, namely D-NA{L-P}VSIPQ, was tested. In this ADNF peptide, all of the amino acids of NAPVSIPQ were in the D-form, except the third proline residue was in the L-form. Cerebral cortical cultures were treated with 1 μ M TTX for 5 days, which is a model of apoptotic death that is relevant to neurodegenerative disease. Cultures treated with the toxin were given various concentrations of D-NA{L-P}VSIPQ. As all L- and all D- amino acid NAPVSIPQ, this mixed D/L peptide D-NA{L-P}VSIPQ retained survival-promoting activity and was effective in cell culture in preventing neuronal cell death in the TTX model.

As illustrates in Figures 3A and 3B, combinations of peptides were also tested. For all combinatorial experiments, the two peptides are given in equimolar amounts. In Figure 3A, the effect of D-NAP and D-SAL was shown to produce a different dose response from that observed with either agent alone. Importantly, there was no apparent attenuation of the survival-promoting activity at higher concentration of peptide. This apparent synergy between the peptides is significant because it indicates that there may be a broader therapeutic range of effective concentrations if both D-peptides are used combinatorially. Similar experiments conducted with both L-SAL and L-NAP resulted in significant loss of efficacy, although synergy was still evident (Figure 3A).

Another series of experiments were conducted to show the effect of combining L- and D-forms of NAP and SAL. As shown in Figure 3B, the use of L-NAP and D-SAL showed full efficacy and high potency in preventing apoptotic death of

neurons treated with TTX. There was no apparent attenuation of the protective activity at high concentrations (> 1 pM) of peptide; *i.e.*, synergy was again evident. In contrast, treatment with D-NAP and L-SAL resulted in full efficacy but attenuation of the survival-promoting activity at concentration > 0.1 pM. These data indicate specificity for combinations of D and L- peptides.

Figure 4 illustrates that ADNF polypeptides can protect against beta amyloid toxicity *in vitro*. PC12 cells (Solomon et al., *Proc. Natl. Acad. Sci. USA* 94: 4109-4112 (1997)) were maintained in DMEM (Dulbecco's modified Eagles medium) supplemented with 8% horse serum, 8% heat-inactivated fetal calf serum, 2mM L-Glutamine, (all purchased from Sigma, Rehovot, Israel), 100 mgr/ml streptomycin and 100 U/l penicillin (Biological Industries, Beit Haemek, Israel). Cultures were maintained at 37°C/5% CO₂ as monolayers in 75 cm² flasks and were split at a 1:12 ratio twice a week. For the beta amyloid (amino acids 25-35) treatment, seeding was at 1.5×10^5 cells/ml on 96-well plates (100 ml/well) in a medium containing: DMEM supplemented with 1% Penicillin/ Streptomycin, 0.5M insulin (Sigma, Rehovot, Israel). Twenty-four hours after the addition of peptides 10-9 M (D-SAL and D-NAP in a 1:1 mixture diluted to final concentration of 1 nM), beta amyloid was added at 2.5 mM and cell viability (metabolic activity) was measured 48 hours later. Metabolic activity was measured by a colorimetric method using a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H tetrazolium (MTS) and an electron coupling reagent phenazine methasulfate. MTS is bio-reduced by the living cells to the Formazan form, that is detected at 490 nm (Promega, Madison WI, USA). Results showed significant activity loss in the presence of the toxin and protection by the peptides D-SAL+D-NAP.

B. *In vivo* experiments

A variety of experimental models were utilized to demonstrate the efficacy of D-NAP and D-SAL in animals. Various routes of administration were employed in both rats and mice.

1. **Fetal alcohol syndrome (FAS) in mice.** A well established model for FAS was used to test the efficacy of ADNF I and ADNF III peptides in mice (Webster *et al.*, *Neurobehav. Tox.* 2:227-234 (1980), incorporated herein by reference). This test is designed to test for efficacy against severe oxidative stress produced from alcohol

administration (Amini *et al.*, *Free Radical Biology and Medicine* 21:357-365 (1996); Schlorff *et al.*, *Alcohol* 17:97-105 (1999)). Fetal death and abnormalities are associated with the generation of free radicals and oxidative damage (Henderson *et al.*, *Alcoholism: Clinical and Experimental Research* 19:714-720 (1995)). The model was chosen in that it allowed for a rapid and relevant evaluation of agents efficacious against severe oxidative stress. Since oxidative stress has been implicated in several neurodegenerative diseases in humans (Cassarmno & Bennett, *Brain Res. Reviews* 29:1-25 (1999)), efficacy in FAS can be of predictive value in the treatment of human disease.

A single injection of 25% ethyl alcohol in saline was given intraperitoneally at 0.030 ml/g body weight to pregnant mice at embryonic day 8. In the first series of experiments, peptides were given 30 minutes prior to the administration of alcohol. Dosages of 2 µg or 20 µg were given. D-NAP or L-NAP (0.5 mg) was dissolved in 50 µl of dimethyl sulfoxide and diluted with filtered (0.22µ) Dulbecco's phosphate buffered saline (DPBS) to a final volume of 5 ml. The injection volume was 200 µl. D-SAL or L-SAL was dissolved in DPBS before administration. The litter mean was used as a single measurement for statistical analysis. The average litter size was 8 and it did not differ among treatment groups.

Evaluation of the surviving fetuses was done on embryonic day 18. Evaluation of efficacy was by the number of surviving fetuses (Figure 5), brain weight (Figure 6A) and total fetal body weight (Figure 6B). As shown in Figure 5, treatment with alcohol resulted in 37% fetal demise in comparison to 6% in controls. Pretreatment with 20 µg D-NAP, D-SAL or L-NAP + D-SAL (20 µg each) significantly reduced the fetal demise rate in comparison to those in the alcohol group ($P < 0.03$). As shown in Figure 6A, of the surviving fetuses whose mother had been treated with alcohol, only those co-treated with L-NAP and D-SAL had significantly greater brain weights in comparison to those in the alcohol group. Similar protective effects of L-NAP and D-SAL were evident as assessed by total fetal weights (Figure 6B).

To test for a critical period of peptide administration that could still produce an effective intervention, L-NAP (20 µg) + L-SAL (20 µg) were administered one hour or three hours after alcohol treatment of pregnant mice at gestational day E8. As shown in Figure 7, post-treatment at 1 hour with NAP + SAL prevented the demises observed with alcohol treatment; however, post-treatment at 3 hours did not result in significant prevention of fetal demise to control levels. In addition, post-treatment with

NAP + SAL (1 hour and 3 hours) prevented the microcephaly (Figure 8), but not the growth restriction associated with FAS.

To demonstrate that D-NAP and D-SAL were effective through oral administration, the peptides were given by gavage (*i.e.*, introducing peptides into the stomach by a tube) to pregnant mice at gestational age day 8. As shown in Figure 9, a significant increase in fetal survival was observed after oral treatment with 40 µg each of D-NAP and D-SAL. This is the first demonstration of an orally active embryo-protecting action of a peptide.

Figures 10A and 10B illustrates effects of oral administration of ADNF polypeptides on pup brain weight and fetal death. Pregnant mice were injected with alcohol as a model for fetal alcohol syndrome according to methods of Webster *et al.* (1980), *supra*. The pregnant mice were injected 25% alcohol at 0.030 ml/g body weight. Peptide was dissolved in phosphate-buffered saline and administered orally by gavage 30 minutes prior to alcohol treatment. D-SAL (all D-amino acids of SALLRSIPA) at 40 µg was found to prevent fetal death as assessed on E18.

2. Apo E knockout mice: developmental behavior assays

Recent studies have demonstrated that the inheritance of the lipid carrier apolipoprotein E4 (ApoE4) is a major risk factor in Alzheimer's disease (Strittmatter & Roses, *Proc. Natl. Acad. Sci. USA* 92:4725-4727 (1995)). These studies, along with the investigations of ApoE-deficient animals indicated that an apolipoprotein E functioning system is required for normal neurodevelopment and function (Masliah *et al.*, *J. Exp. Neurol.* 136:107-122 (1995)). The acquisition of developmental milestones of behavior requires appropriate synapse formation and proper brain conductivity (Altman *et al.*, *Anim. Behav.* 23:896-920 (1975)). ApoE-deficient animals have been shown to be developmentally retarded (Gozes *et al.*, *J. Neurobiol.* 33: 329-342 (1997), incorporated herein by reference) offer a test system for the *in vivo* effects of putative neurotrophic substances, such as D-SAL and D-NAP.

Newborn animals were tested for the onset neurobehavioral developmental milestones as previously described (Gozes *et al.*, *J. Neurobiol.* 33: 329-342 (1997); Bassan *et al.*, *J. Neurochem.* 72: 1283-1293 (1999)). For these experiments, animals were treated either by oral application, or subcutaneous injection of D-SAL + D-NAP. Peptides (0.5 mg each) were dissolved in 0.01M acetic acid (30 microliters). For both

applications, 0.5 microgram of each of the test drugs were delivered; for the oral application (sublingual), in 10 microliter saline and for the injection in 20 microliters. This protocol was used for the first 4 days of life. From day 5-10, the amount of the peptides and the solution volume was doubled. From day 11-14, the amount of peptide was 2 microgram each in 40 microliter (oral) and 80 microliter (injection). Tests performed daily included cliff avoidance, negative geotaxis, placing and righting behaviors. Both subcutaneous and oral administration of D-NAP and D-SAL were compared. As shown in Figure 11, the slowest responders for cliff avoidance were the apoE knockout animals. This confirms previous studies which show that the behavioral developmental and learning is delayed in these animals in comparison to control animals (Gozes *et al.*, *J. Neurobiol.* 33:329-342 (1997); Bassan *et al.*, *J. Neurochem.* 72:1283-1293 (1999)). Administration of D-NAP + D-SAL by either subcutaneous injection or oral administration resulted in significant increases in the behavioral score, indicative of a more rapid acquisition of this developmental milestone. Similar effects were observed for negative geotaxis (Figure 12) and placing behavior (Figure 13).

More detailed evaluation of the results is as follows. In the following, one way analysis of variance with multiple comparison of the means (Student-Newman-Keuls method) were used for statistical comparisons.

1. Figure 11 (cliff avoidance): the difference between apoE-deficient mice and control animals was apparent only on the fifth day of life ($P < 0.001$). Injection of D-peptides to control, resulted in no effect, while injection to the deficient mice resulted in an effect only on the third day. Oral application resulted in a significant improvements only on the first day, in the deficient mice.

2. Figure 12 (negative geotaxis): While there was no difference on the first day between control and apoE-deficient (with perhaps a difference on day three, $P < 0.006$), treatment of the latter (injection or oral) resulted in significant improvements with injection on days 1, 2, 4 and 5, and with oral treatment on days 1 and 5. ($P < 0.001$).

3. Figure 13 (placing): The difference between apoE-deficient mice and control animals was apparent only on the first day of life ($P < 0.001$). Similarly, oral application of the peptide mixture was efficient in enhancing the response only on the first day of life.

3. AF64A cholinotoxicity in adult rats

Another focus of the present invention are the neuroprotective properties of the D-SAL and D-NAP in animals exposed to the cholinotoxin, ethylcholine aziridium (AF64A), a blocker of choline uptake (Fisher *et al.*, *Neurosci. Lett.* 102:325-331 (1989)).

- 5 An intact cholinergic system is required for normal brain function, whereas Alzheimer's disease is associated with the death of cholinergic cells (Brumback & Leech, *J. Okla. State Med. Assoc.* 87:103-111 (1994)). Rats treated with AF64A provide an accepted model for testing *in vivo* efficacy of cholinergic-enhancing drugs.

- 10 Although the identity of the ADNF-dependent neurons has not been fully characterized, previous studies indicated that some cholinergic neurons are among those affected (Gozes *et al.*, *Brain Res. Dev.* 99:167-175 (1997)). In this context, ApoE-deficient mice (described above) exhibited reduced choline acetyl transferase activity (Gordon *et al.*, *Neurosci. Lett.* 199:1-4 (1995); Gozes *et al.*, *J. Neurobiol.* 33:329-342 (1997)) and treatment with L-NAP significantly increased cholinergic function to control levels (Bassan *et al.*, *J. Neurochem.* 72:1283-1293 (1999)) while L-SAL treatment was less effective.

- Rats (male Wistar, 300-350g) were subjected to two daily tests in a water maze, including a hidden platform (Morris, *J. Neurosci. Methods* 11:47-60 (1984) and Gordon *et al.*, *Neurosci. Lett.* 199:1-4 (1995); Gozes *et al.*, *J. Neurobiol.* 33:329-342 (1997)). Every day for the first test, both the platform and the animal were situated in a new location with regard to the pool (with the pool being immobile). The experiment was performed as follows: the animal was positioned on the platform for 0.5 minutes then placed in the water. The time required to reach the platform (indicative of learning and intact reference memory) was measured (first test). After 0.5 minute on the platform, the animal was placed back in the water (in the previous position) for an additional second test and search for the hidden platform (retained in the previous position). The time required to reach the platform in the second trial was recorded, indicative of short-term (working) memory. All measurements were performed using the computerized video-assisted HVS water maze system (HVS Image Ltd. Hampton, UK). Animals were tested for four days to eliminate random memory defective animals. The best performers were injected i.c.v. at a rate of 0.21 μ l/min. with the cholinotoxin ethylcholine aziridium (AF64A, 3 nmol/2 μ l/side), control animals received an injection of saline (Gozes *et al.*, *Proc. Natl. Acad. Sci. USA* 93:427-432 (1996)).

Animals were allowed to recover for one week, followed by daily exposure to three micrograms of D-SAL + three micrograms of D-NAP, in 20 microliter saline, applied orally on the tongue. After a week of oral peptide application, the animals were subjected to two daily tests in the water maze (as above). During the test-period, animals were also given an oral administration of peptide or vehicle (carrier) an hour before the daily test. It was previously shown that AF64A-treated animals exhibit learning and memory deficits in the Morris water maze test (Gozes *et al.*, *Proc. Natl. Acad. Sci. USA* 93:427-432 (1996)). Here, AF64 A-treated rats subjected to oral application of D-NAP + D-SAL exhibited a decreased latency in finding the hidden platform, indicative of improved reference memory (Figure 14A, first daily test). Furthermore, the same rats exhibited improved working memory in the second daily test (Figure 14B). These data indicate that oral administration of a combination of D-SAL and D-NAP resulted in significant increases in learning and memory in animals with chemically induced cholinergic impairment.

Figure 14C illustrates the effect of oral administration of D-SALLRSIPA alone on learning and memory in rats treated with the cholinotoxin AF-64A. Rats were treated with the cholinotoxin AF-64A and D-SALLRSIPA as described in Gozes *et al.*, *J. Pharmacol. Exp. Therap.* 293: 1091-1098 (2000), except that D-SALLRSIPA was delivered to AF64A-treated rats was as follows: 10 microgram D-SALLRSIPA (D-SAL) per rat (250-300 g) per day in 50 microliter saline under the tongue, using a micropipette. Peptides were applied once daily for three days, a week after the AF64A lesion. After a 2-day cessation, peptides were applied once daily for another 5 days and tested from day three on. Following an additional two-day cessation, peptides were applied again daily for two days and tested in the Morris water maze. The graph shows the results of the 5 day testing. In each day the animals were subjected to two consecutive tests and results are a summation of the two daily tests. Significance (one way ANOVA with Student-Neuman-Kuels multiple comparison of means test) is as follows.

Day 1: $P < 0.04$ D-SALLRSIPA-AF64A vs. AF64A;

Day 2: $P < 0.04$ AF64A vs. control (sham operated), SALLRSIPA treatment was not significantly different from either AF64A animals or control, suggesting some improvement;

Day 3: No difference;

Day 4: No difference; and

Day 5: t-test: $P < 0.04$ D-SALLRSIPA-AF64A vs. AF64A.

These results suggest that D-SALLRSIPA (D-SAL) is effective on its own.

4. Memory improvements in ApoE-deficient mice:

Memory deficits and cholinergic impairments have been described in adult ApoE-deficient mice. These deficits may mimic the conditions found in people that are homozygous for apolipoprotein E4, a condition that in which patients are more prone to an early onset of Alzheimer's disease, in contrast to people carrying the E2 or E3 alleles (Gordon *et al.*, *Neurosci. Lett.* 199:1-4 (1995)). A week after cessation of treatment, cognitive functions were assessed in the Morris water maze. Improvements of cognitive functions were observed a week after cessation of the 2-week daily D-SAL-D-NAP treatment, *i.e.* in 21-day-old mice exposed to a 5-day training protocol (Figure 15). Short-term memory processes were examined by performance in the water maze, measuring the time required to find the hidden platform in the second of two daily trials. The platform location and the starting point in which the animal was placed in the water were held constant within each pair of daily trials, but both locations were changed every day. On the second test of the first trial day, the ApoE-deficient mice were significantly retarded as compared to controls ($P < 0.04$) and improved after oral application of D-SAL + D-NAP, with most of the treated animals finding the platform at a latency of ≤ 20 sec.

Figures 16A and 16B illustrate the first test and second test, respectively, of Morris water maze test results in apolipoprotein E-deficient mice. Experiments were performed following injections of a mixture of D-NAP-D-SAL with an injection protocol and Morris water maze as described in Gozes *et al.*, *J. Pharmacol. Exp. Therap.* 293: 1091-1098 (2000)). Results showed significant improvements on day 1 and day 2 (first daily test, and on day three, second daily test)- $P < 0.05$.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. For example, any one or more of the features of the

previously described embodiments can be combined in any manner with one or more features of any other embodiments in the present invention. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of

5 equivalents.

WHAT IS CLAIMED IS:

- 1 1. An Activity Dependent Neurotrophic Factor I (ADNF I)
2 polypeptide, the ADNF I polypeptide comprising an active core site having the following
3 amino acid sequence:
4 Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1),
5 wherein the active core site comprises at least one D-amino acid.
- 1 2. The ADNF I polypeptide of claim 1, wherein either an N-terminal
2 amino acid or a C-terminal amino acid of the active core site is a D-amino acid.
- 1 3. The ADNF I polypeptide of claim 1, wherein both N-terminal and
2 C-terminal amino acids of the active core site are D-amino acids.
- 1 4. The ADNF I polypeptide of claim 1, wherein the active core site
2 comprises all D-amino acids.
- 1 5. The ADNF I polypeptide of claim 1, wherein the ADNF I
2 polypeptide is Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1).
- 1 6. The ADNF I polypeptide of claim 5, wherein the ADNF I
2 polypeptide comprises all D-amino acids.
- 1 7. The ADNF I polypeptide of claim 1, wherein the ADNF I
2 polypeptide is selected from the group consisting of:
3 Val-Leu-Gly-Gly-Gly- Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:14);
4 Val-Glu-Glu-Gly-Ile-Val-Leu-Gly-Gly-Gly-Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-
5 Ala (SEQ ID NO:15);
6 Leu-Gly-Gly-Gly- Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:16);
7 Gly-Gly-Gly-Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:17);
8 Gly-Gly-Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:18); and
9 Gly- Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:19).
- 1 8. The ADNF I polypeptide of claim 1, wherein the ADNF I
2 polypeptide comprises up to about 20 amino acids at each of an N-terminus and a C-
3 terminus of the active core site.

- 1 9. The ADNF I polypeptide of claim 8, wherein both N-terminal and
2 C-terminal amino acids of the ADNF I polypeptide are D-amino acids.
- 1 10. An Activity Dependent Neurotrophic Factor III (ADNF III)
2 polypeptide, the ADNF III polypeptide comprising an active core site having the
3 following amino acid sequence:
4 Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2),
5 wherein the active core site comprises at least one D-amino acid.
- 1 11. The ADNF III polypeptide of claim 10, wherein either an N-
2 terminal amino acid or an C-terminal amino acid of the active core site is a D-amino acid.
- 1 12. The ADNF III polypeptide of claim 10, wherein both N-terminal
2 and C-terminal amino acids of the active core site are D-amino acids.
- 1 13. The ADNF III polypeptide of claim 10, wherein the active core site
2 comprises all D-amino acids.
- 1 14. The ADNF III polypeptide of claim 10, wherein the ADNF III
2 polypeptide is Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2).
- 1 15. The ADNF III polypeptide of claim 14, wherein the ADNF III
2 polypeptide comprises all D-amino acids.
- 1 16. The ADNF III polypeptide of claim 10, wherein the ADNF III
2 polypeptide is selected from the group consisting of:
3 Gly-Gly-Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:20);
4 Leu-Gly-Gly-Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln-Gln-Ser (SEQ ID NO:21);
5 Leu-Gly-Leu-Gly-Gly-Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln-Gln-Ser (SEQ ID
6 NO:22); and
7 Ser-Val-Arg-Leu-Gly-Leu-Gly-Gly-Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln-Gln-Ser
8 (SEQ ID NO:23).
- 1 17. The ADNF III polypeptide of claim 10, wherein the ADNF III
2 polypeptide comprises up to about 20 amino acids at each of an N-terminus and an C-
3 terminus of the active core site.

- 1 18. The ADNF III polypeptide of claim 17, wherein both N-terminal
2 and C-terminal amino acids of the ADNF I polypeptide are D-amino acids.
- 1 19. A pharmaceutical composition comprising a pharmaceutically
2 acceptable excipient and an Activity Dependent Neurotrophic Factor (ADNF)
3 polypeptide, wherein the ADNF polypeptide is a member selected from the group
4 consisting of:
5 (a) an ADNF I polypeptide comprising an active core site having the
6 following amino acid sequence:
7 Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1);
8 (b) an ADNF III polypeptide comprising an active core site having the
9 following amino acid sequence:
10 Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2); and
11 (c) a mixture of the ADNF I polypeptide of part (a) and the ADNF III
12 polypeptide of part (b);
13 wherein at least one of the ADNF I polypeptide and the ADNF III polypeptide comprises
14 an active core site comprising at least one D-amino acid.
- 1 20. The pharmaceutical composition of claim 19, wherein the ADNF
2 polypeptide is an ADNF I polypeptide and wherein the active core site of the ADNF I
3 polypeptide comprises at least one D-amino acid.
- 1 21. The pharmaceutical composition of claim 20, wherein both N-
2 terminal and C-terminal amino acids of the active core site of the ADNF I polypeptide are
3 D-amino acids.
- 1 22. The pharmaceutical composition of claim 20, wherein the active
2 core site of the ADNF I polypeptide comprises all D-amino acids.
- 1 23. The pharmaceutical composition of claim 20, wherein the ADNF I
2 polypeptide is Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1).
- 1 24. The pharmaceutical composition of claim 23, wherein the ADNF I
2 polypeptide comprises all D-amino acids.

1 25. The pharmaceutical composition of claim 19, wherein the ADNF
2 polypeptide is an ADNF III polypeptide and wherein the active core site of the ADNF III
3 polypeptide comprises at least one D-amino acid.

1 26. The pharmaceutical composition of claim 25, wherein both N-
2 terminal and C-terminal amino acids of the active core site of the ADNF III polypeptide
3 are D-amino acids.

1 27. The pharmaceutical composition of claim 25, wherein the active
2 core site of the ADNF III polypeptide comprises all D-amino acids.

1 28. The pharmaceutical composition of claim 25, wherein the ADNF
2 III polypeptide is Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2).

1 29. The pharmaceutical composition of claim 28, wherein the ADNF
2 III polypeptide comprises all D-amino acids.

1 30. The pharmaceutical composition of claim 19, wherein the ADNF
2 polypeptide is a mixture of an ADNF I polypeptide of part (a) and an ADNF III
3 polypeptide of part (b) and wherein at least one of the ADNF I polypeptide and the
4 ADNF III polypeptide comprises an active core site comprising at least one D-amino
5 acid.

1 31. The pharmaceutical composition of claim 30, wherein both N-
2 terminal and C-terminal amino acids of the active core site of the ADNF I polypeptide are
3 D-amino acids, and wherein both N-terminal and C-terminal amino acids of the active
4 core site of the ADNF III polypeptide are D-amino acids.

1 32. The pharmaceutical composition of claim 30, wherein the active
2 core site of the ADNF I polypeptide comprises all D-amino acids, and wherein the active
3 core site of the ADNF III polypeptide comprises all D-amino acids.

1 33. The pharmaceutical composition of claim 30, wherein the ADNF I
2 polypeptide is Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1) and wherein the
3 ADNF III polypeptide is Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2).

1 34. The pharmaceutical composition of claim 33, wherein the ADNF I
2 polypeptide comprises all D-amino acids and wherein the ADNF III polypeptide
3 comprises all D-amino acids.

1 35. The pharmaceutical composition of claim 30, wherein the ADNF
2 polypeptide I comprises all D-amino acids and wherein the ADNF III polypeptide
3 comprises all L-amino acids.

1 36. The pharmaceutical composition of claim 30, wherein the ADNF I
2 polypeptide comprises all L-amino acids, and wherein the ADNF III polypeptide
3 comprises all D-amino acids.

1 37. The pharmaceutical composition of claim 19, wherein the
2 composition is formulated for intranasal, intraperitoneal, subcutaneous, gavage,
3 sublingual, intravenous, or oral administration.

1 38. The pharmaceutical composition of claim 19, wherein the
2 composition is formulated for oral administration.

1 39. The pharmaceutical composition of claim 22, wherein the
2 composition is formulated for oral administration.

1 40. The pharmaceutical composition of claim 27, wherein the
2 composition is formulated for oral administration.

1 41. The pharmaceutical composition of claim 32, wherein the
2 composition is formulated for oral administration.

1 42. A method for reducing neuronal cell death, the method comprising
2 contacting the neuronal cells with an Activity Dependent Neurotrophic Factor (ADNF)
3 polypeptide in an amount sufficient to prevent neuronal cell death, wherein the ADNF
4 polypeptide is a member selected from the group consisting of:

5 (a) an ADNF I polypeptide comprising an active core site having the
6 following amino acid sequence:

7 Ser-Ala-Leu-L_{eu}-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1);

8 (b) an ADNF III polypeptide having the following amino acid sequence:

9 Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2); and
10 (c) a mixture of the ADNF I polypeptide of part (a) and the ADNF III
11 polypeptide of part (b);
12 wherein at least one of the ADNF I polypeptide and the ADNF III polypeptide comprises
13 an active core site comprising at least one D-amino acid.

1 43. The method of claim 42, wherein the ADNF polypeptide is an
2 ADNF I polypeptide and wherein the active core site of the ADNF I polypeptide
3 comprises all D-amino acids.

1 44. The method of claim 43, wherein the ADNF I polypeptide is Ser-
2 Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1).

1 45. The method of claim 44, wherein the ADNF I polypeptide
2 comprises all D-amino acids.

1 46. The method of claim 42, wherein the ADNF polypeptide is an
2 ADNF III polypeptide and wherein the ADNF III polypeptide comprises all D-amino
3 acids.

1 47. The method of claim 46, wherein the ADNF III polypeptide is Asn-
2 Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2).

1 48. The method of claim 47, wherein the ADNF III polypeptide
2 comprises all D-amino acids.

1 49. The method of claim 42, wherein the ADNF polypeptide is a
2 mixture of an ADNF I polypeptide of part (a) and an ADNF III polypeptide of part (b)
3 and wherein the ADNF I polypeptide and the ADNF III polypeptide both comprise all D-
4 amino acids.

1 50. The method of claim 49, wherein the ADNF I polypeptide is Ser-
2 Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1) and wherein the ADNF III polypeptide
3 is Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2).

1 51. The method of claim 42, wherein the ADNF polypeptide is a
2 mixture of an ADNF I polypeptide of part (a) and an ADNF III polypeptide of part (b),

- 3 and wherein the ADNF I polypeptide comprises all D-amino acids and wherein the
4 ADNF III polypeptide comprises all L-amino acids.

1 52. The method of claim 42, wherein the ADNF polypeptide is a
2 mixture of an ADNF I polypeptide of part (a) and an ADNF III polypeptide of part (b),
3 and wherein the ADNF I polypeptide comprises all L-amino acids and wherein the ADNF
4 III polypeptide comprises all D-amino acids.

1 53. The method of claim 42, wherein the neuronal cells are selected
2 from the group consisting of spinal cord neurons, hippocampal neurons, cerebral cortical
3 neurons and cholinergic neurons.

1 54. The method of claim 42, wherein the neuronal cell death is in a
2 patient infected with immunodeficiency virus.

1 55. The method of claim 54, wherein the immunodeficiency virus is a
2 human immunodeficiency virus.

1 56. The method of claim 42, wherein the neuronal cell death is
2 associated with excitotoxicity induced by N-methyl-D-aspartate stimulation.

1 57. The method of claim 42, wherein the neuronal cell death is induced
2 by the beta-amyloid peptide in a patient afflicted with Alzheimer's disease.

1 58. The method of claim 42, wherein the neuronal cell death is induced
2 by cholinergic blockade in a patient afflicted with Alzheimer's disease, the cholinergic
3 blockade resulting in learning impairment.

1 59. A method for treating oxidative stress in a patient, the method
2 comprising administering to the patient an Activity Dependent Neurotrophic Factor
3 (ADNF) polypeptide in an amount sufficient to reduce oxidative stress, wherein the
4 ADNF polypeptide is a member selected from the group consisting of:

5 (a) an ADNF I polypeptide comprising an active core having the following
6 amino acid sequence:

7 Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1);

8 (b) an ADNF III polypeptide having the following amino acid sequence:

9 Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2); and

10 (c) a mixture of the ADNF I polypeptide of part (a) and the ADNF III
11 polypeptide of part (b);
12 wherein at least one of the ADNF I polypeptide and the ADNF III polypeptide comprises
13 an active core site comprising at least one D-amino acid.

1 60. The method of claim 59, wherein the ADNF polypeptide is an
2 ADNF I polypeptide, and wherein the active core site of the ADNF I polypeptide
3 comprises all D-amino acids.

1 61. The method of claim 60, wherein the ADNF I polypeptide is Ser-
2 Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1).

1 62. The method of claim 61, wherein the ADNF I polypeptide
2 comprises all D-amino acids.

1 63. The method of claim 59, wherein the ADNF polypeptide is an
2 ADNF III polypeptide, and wherein the ADNF III polypeptide comprises all D-amino
3 acids.

1 64. The method of claim 63, wherein the ADNF III polypeptide is Asn-
2 Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2).

1 65. The method of claim 64, wherein the ADNF III polypeptide
2 comprises all D-amino acids.

1 66. The method of claim 59, wherein the ADNF polypeptide is a
2 mixture of an ADNF I polypeptide of part (a) and an ADNF III polypeptide of part (b)
3 and wherein the ADNF I polypeptide and the ADNF III polypeptide both comprise all D-
4 amino acids.

1 67. The method of claim 66, wherein the ADNF I polypeptide is Ser-
2 Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1) and wherein the ADNF III polypeptide
3 is Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2).

1 68. The method of claim 59, wherein the ADNF polypeptide is a
2 mixture of an ADNF I polypeptide of part (a) and an ADNF III polypeptide of part (b)

3 and wherein the ADNF I polypeptide comprises all D-amino acids and wherein the
4 ADNF III polypeptide comprises all L-amino acids.

1 69. The method of claim 59, wherein the ADNF polypeptide is a
2 mixture of an ADNF I polypeptide of part (a) and an ADNF III polypeptide of part (b)
3 and wherein the ADNF I polypeptide comprises all L-amino acids and wherein the ADNF
4 III polypeptide comprises all D-amino acids.

1 70. A method for reducing a condition associated with fetal alcohol
2 syndrome in a subject who is exposed to alcohol *in utero*, the method comprising
3 administering to the subject an ADNF polypeptide in an amount sufficient to reduce the
4 condition associated with fetal alcohol syndrome, wherein the ADNF polypeptide is a
5 member selected from the group consisting of:

6 (a) an ADNF I polypeptide comprising an active core site having the
7 following amino acid sequence:

8 Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1);

9 (b) an ADNF III polypeptide having the following amino acid sequence:

10 Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2); and

11 (c) a mixture of the ADNF I polypeptide of part (a) and the ADNF III
12 polypeptide of part (b);

13 wherein at least one of the ADNF I polypeptide and the ADNF III polypeptide comprises
14 an active core site comprising at least one D-amino acid.

1 71. The method of claim 70, wherein the ADNF polypeptide is an
2 ADNF I polypeptide, and wherein the ADNF I polypeptide comprises all D-amino acids.

1 72. The method of claim 71, wherein the ADNF I polypeptide is Ser-
2 Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1).

1 73. The method of claim 72, wherein the ADNF I polypeptide
2 comprises all D-amino acids.

1 74. The method of claim 70, wherein the ADNF polypeptide is an
2 ADNF III polypeptide, and wherein the ADNF III polypeptide comprises all D-amino
3 acids.

1 75. The method of claim 74, wherein the ADNF III polypeptide is Asn-
2 Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2).

1 76. The method of claim 70, wherein the ADNF polypeptide is a
2 mixture of an ADNF I polypeptide of part (a) and an ADNF III polypeptide of part (b)
3 and wherein the ADNF I polypeptide and the ADNF III polypeptide both comprise all D-
4 amino acids.

1 77. The method of claim 76, wherein the ADNF I polypeptide is Ser-
2 Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (SEQ ID NO:1) and wherein the ADNF III polypeptide
3 is Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln (SEQ ID NO:2).

1 78. The method of claim 70, wherein the ADNF polypeptide is a
2 mixture of an ADNF I polypeptide of part (a) and an ADNF III polypeptide of part (b)
3 and wherein the ADNF I polypeptide comprises all D-amino acids and wherein the
4 ADNF III polypeptide comprises all L-amino acids.

1 79. The method of claim 70, wherein the ADNF polypeptide is a
2 mixture of an ADNF I polypeptide of part (a) and an ADNF III polypeptide of part (b)
3 and wherein the ADNF I polypeptide comprises all L-amino acids and wherein the ADNF
4 III polypeptide comprises all D-amino acids.

1 80. The method of claim 70, wherein the condition is selected from the
2 group consisting of: a decreased body weight of a subject; a decreased brain weight of the
3 subject; a decreased level of VIP mRNA of a subject; and death of a subject *in utero*.

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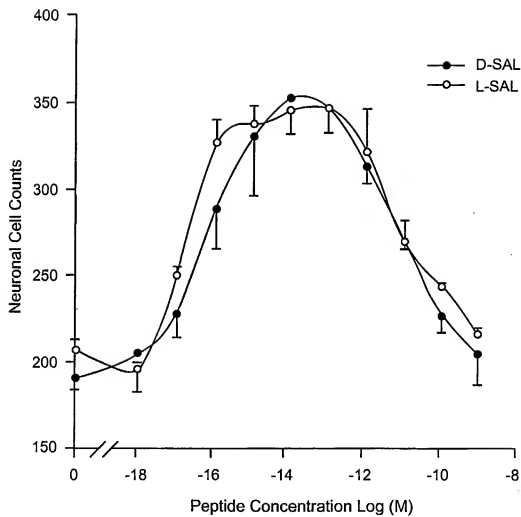


FIG. 1

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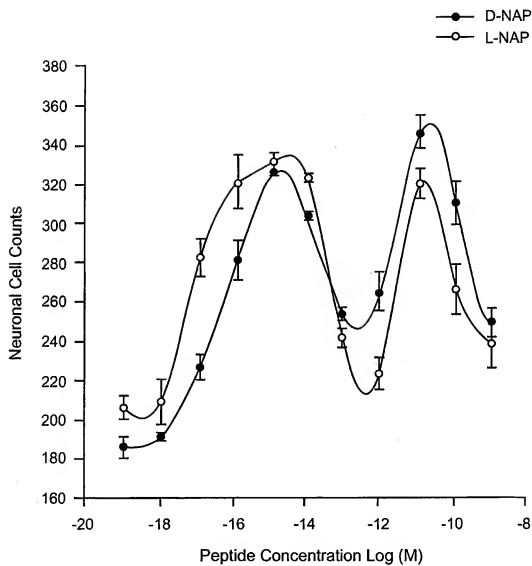


FIG. 2A

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E1066: Effect of D-NA{L-P} on SPF in TTX-treated
Mixed Cerebral Cortical Cultures

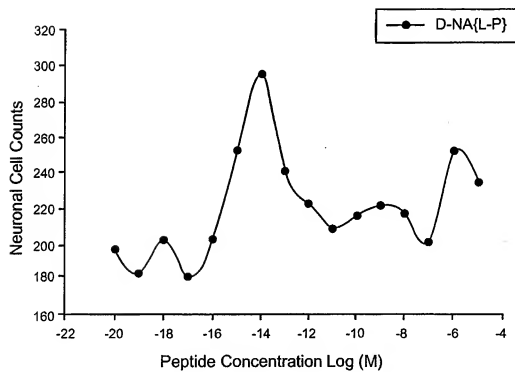


FIG. 2B

4 / 21

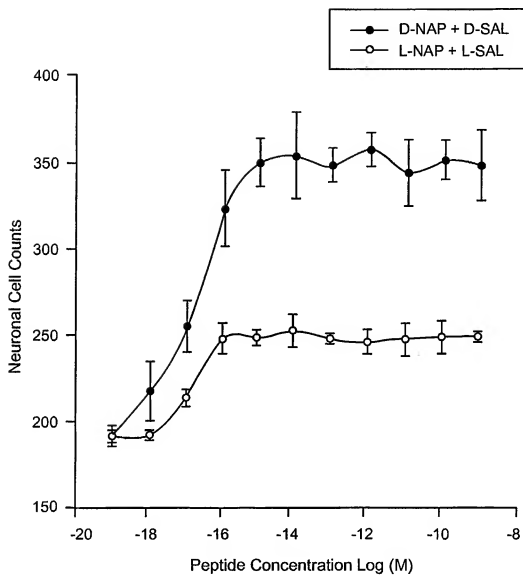


FIG. 3A

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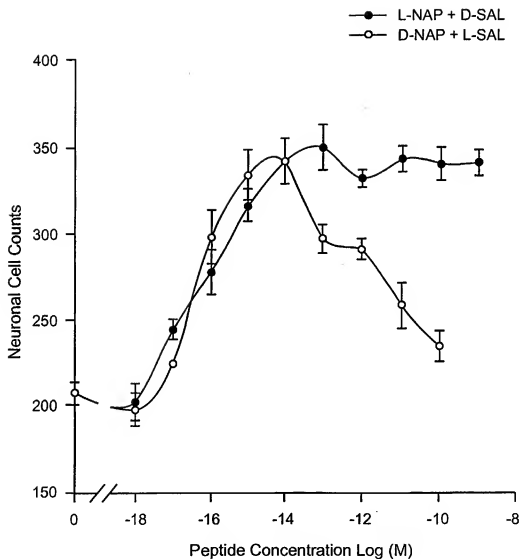


FIG. 3B

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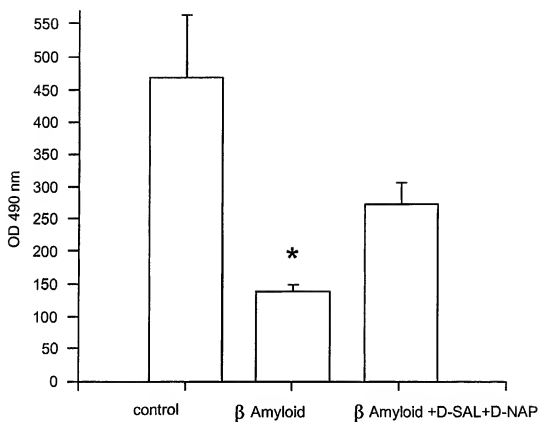


FIG. 4

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FIG. 5

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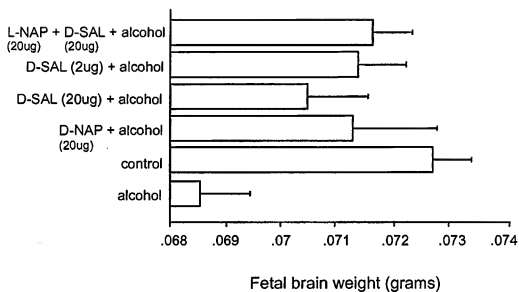


FIG. 6A

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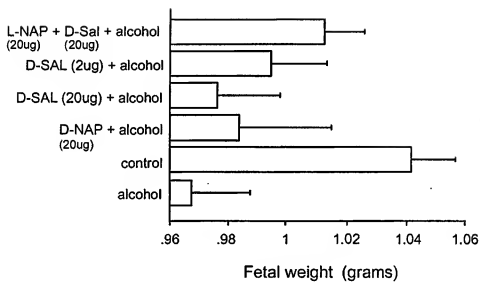


FIG. 6B

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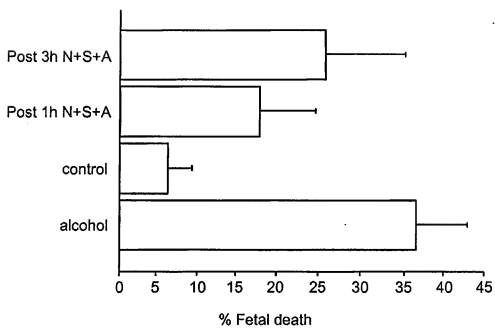


FIG. 7

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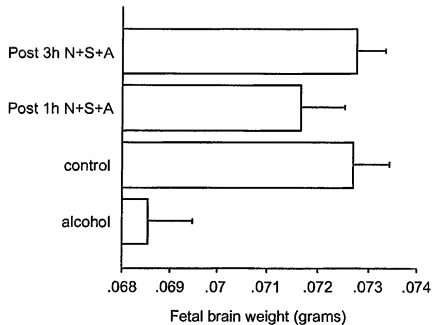


FIG. 8

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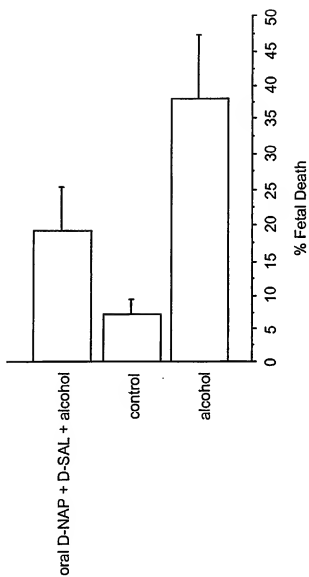


FIG. 9

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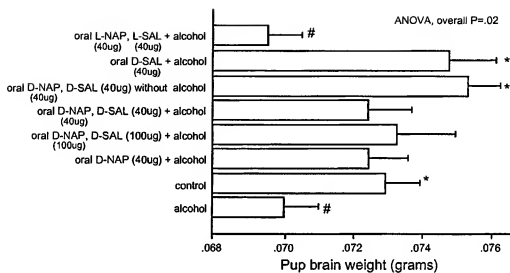


FIG. 10A

*Oral D-SAL, D-NAP+D-SAL (40 or 100ug)
prevent alcohol-induced fetal demise*

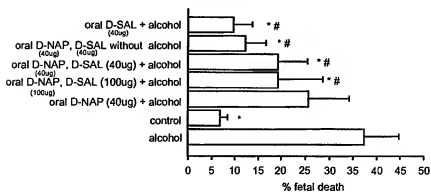


FIG. 10B

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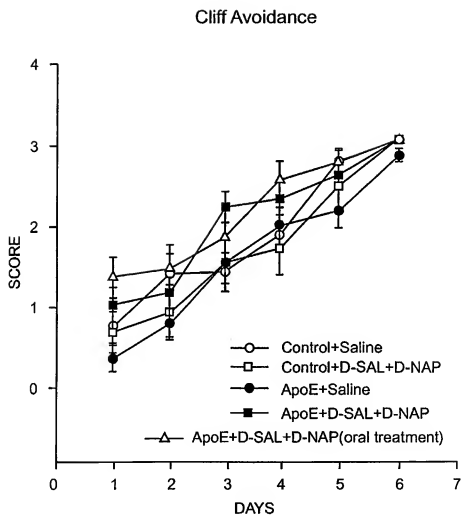


FIG. 11

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Negative geotaxis

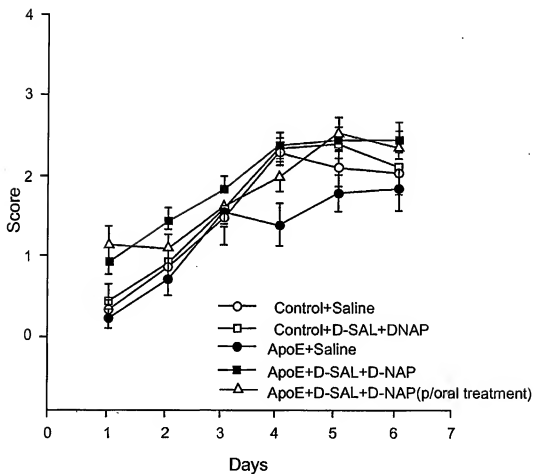


FIG. 12

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Placing

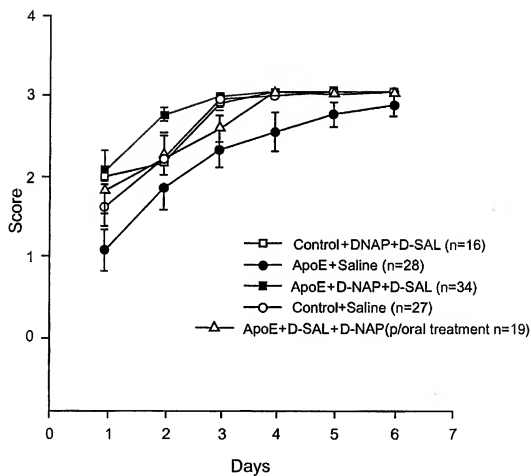


FIG. 13

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First test

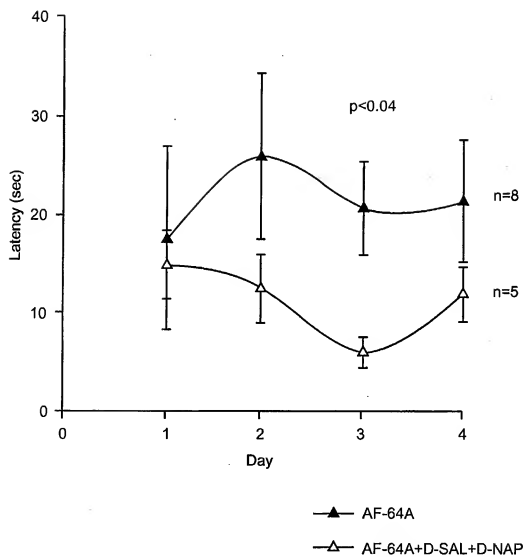


FIG. 14A

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Second test

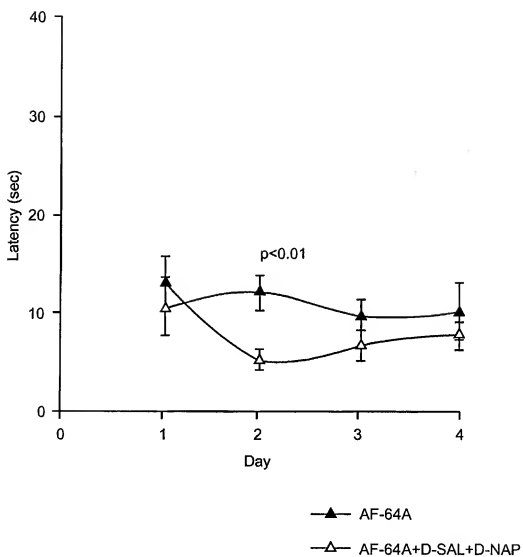


FIG.14B

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First and second swim

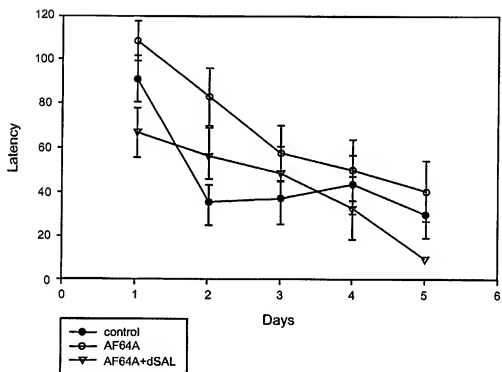


FIG. 14C

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Second test

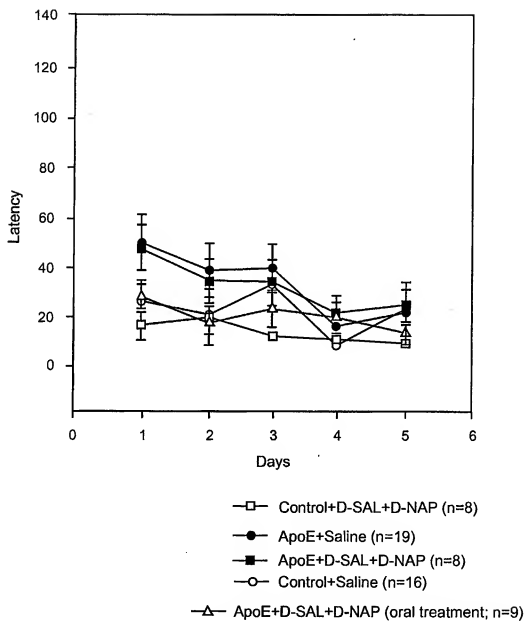


FIG. 15

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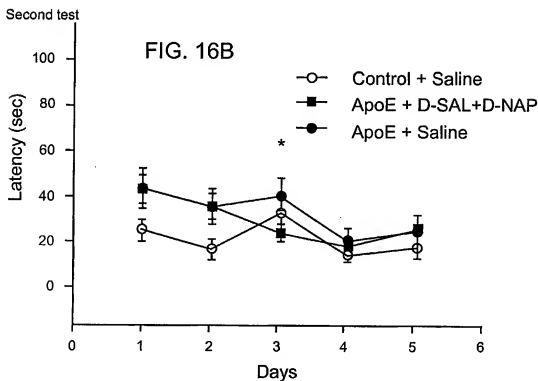
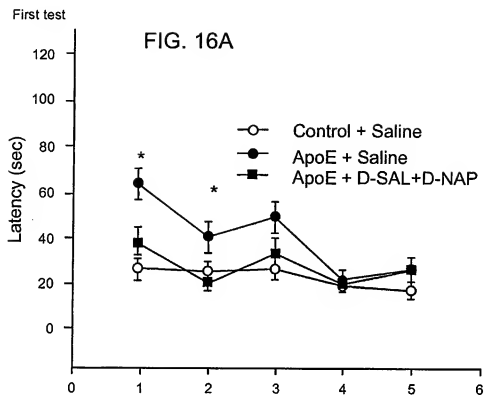


EXHIBIT E

Encephalomyelitis Experimental Autoimmune (EAE) was induced in female mice (eight weeks old, 18-20 g body weight, C57BL/6) by subcutaneous injection (into the thigh) of an emulsion (200 μ l) containing 300 μ g myelinoligodendrocyte glycoprotein (MOG) 35-55 peptide in complete Freund's adjuvant (Sigma) supplemented by 400 μ g *Mycobacterium tuberculosis*. A similar boost injection was administered a week later in the opposite thigh. In addition, the mice were injected with 300ng Pertussis Toxin in saline (100 μ l) immediately after the first injection and 48 hours later. The disease was inflicted in ~45 mice that were divided into three groups, control, injected daily, ip with 0.1 ml saline/mouse, 2 μ g all D-amino acid SALLRSIPA (D-SAL)/mouse/day or 20 μ g D-SAL/mouse/day. The mice were treated from the onset of clinical signs (~10 days from the first injection of the toxins). Only mice that showed clinical symptoms were treated. Mice that did not show clinical disease outcome or died w/o any disease symptoms were excluded from the evaluations.

Immunized mice were observed daily for clinical signs and scored on a 0-6 scale, with 0.5 gradations for intermediate scores, as follows: 0, normal without clinical signs; 1, complete limp tail; 2, abnormal righting reflex; 3, Ataxia; 4, complete hindlimb paralysis; 5, Full paralysis; and 6, death.

Saline-treated EAE mice manifested progressive clinical impairment, reaching almost a score of 5 at the maximum. In contrast, in D-SAL-treated mice the disease seemed to progress with a shallower slope and those treated with 2 μ g/day/mouse did not reach an apparent same severity as the saline-treated mice (Figure - EAE8).

